Drug Transporters of the Fungal Wheat Pathogen

Mycosphaerella graminicola

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This thesis is dedicated to: ***my wife and best friend, Mahnaz***

my parental family
especially my mother

and my lovely niece, Aylin

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Contents

Chapter 1

General introduction and outline

Wheat

Since early history, wheat (*Triticum aestivum* L.) is a main source of food for human beings. It is the oldest and most widely grown food crop since 10,000-8,000 B.C. (Orth and Shellenberger, 1988). The importance of wheat in Iran is emphasized by the Iranian religious prophet Asho Zartosht (630-550 B.C.) of Zoroastrianism who stated that “anybody who grows wheat multiplies the truth”. At present, wheat is still the most important cereal crop in the world. It occupies 17% of the cultivated land and is the main food for 35% of the world population. Wheat provides 20% of the daily intake of calories by humans. In Iran, wheat is the most important agricultural crop, and bread as its main product provides over 53% of daily consumed calories. The size of global and Iranian areas of wheat cultivation is estimated 228 and 6.3 million hectare with an average yield of 2,634 and 1,595 kg per hectare, respectively.

As a consequence of global population growth, the demand for wheat will grow faster than for any other major crop. The forecasted global demand for wheat in the year 2020 varies between 840 (Rosegrant et al., 1995) and 1050 million tons (Kronstad, 1998). To reach this target, global production needs an annual increase of 1.6 to 2.6%. Plant pathogens, insects and weeds are the major threats that can prevent this required increase since these pests can potentially reduce yield by 50% without crop protection.

Septoria leaf blotch

Two major septoria diseases on wheat are septoria tritici blotch (syn. septoria leaf blotch, speckled leaf blotch) caused by *Septoria tritici* (teleomorph: *Mycosphaerella graminicola*), and septoria nodorum blotch (syn. septoria glume blotch) caused by *Stagonospora nodorum* (teleomorph: *Phaeosphaeria nodorum*). Total losses of wheat yield due to septoria diseases worldwide are estimated over nine million metric tons, and annual yield losses during outbreaks can range from 30 to 53% (Babadoost and Herbert, 1984; Eyal et al., 1987; Palmer and Skinner, 2002). In the last decades, septoria diseases occur more frequently, especially in heavily fertilized crops where plant show

dense foliage, and in areas where semi-dwarf and rust-resistant wheat cultivars are grown. Losses are most dramatic when epidemics develop before the onset of heading (Wiese, 1987). Septoria tritici blotch is regarded as the major threat of wheat crops in Europe and causes serious losses of both bread and durum wheat. It also reduces yield and quality in many other parts of the world where wheat is grown. This disease has spread globally across a wide range of geographical niches, and has gradually emerged as one of the most damaging foliar diseases of wheat, especially in areas with high rainfall during the growing season. The disease is characterized by the appearance of typical necrotic blotches on leaves that contain black or dark brown pycnidia containing the asexual pycnidiospores (Eyal et al., 1985). The most common way the pathogen penetrates the plant is via the stomata (Kema et al., 1996b). Symptoms develop throughout the growing season on all aerial plant parts. The first symptoms on wheat leaves are irregular chlorotic lesions that usually appear 5-6 days after infection, but this timepoint may vary depending the cultivar and environmental conditions during the infection process. Necrotic lesions develop at the chlorotic sites after 8-12 days at 18-24 °C and high relative humidity. Initially, in the necrotic lesions the tissue collapses and has a grayish-green appearance. Pycnidia, ranging in color from light to dark brown, develop in the necrotic lesions on both the upper and lower surface of the leaves. The primary inoculum for infection might be either the air-borne ascospores (travel over long distances within and between wheat fields) released from pseudothecia or the rain-splashed pycnidiospores (dispersible over short distances within the crop) released from pycnidia in crop debris and on volunteer wheat or other susceptible grass species. Throughout the growing season, pycnidiospores are produced in multiple cycles, and these are the secondary source of inoculum for infection.

Mycosphaerella graminicola

Mycosphaerella is the largest genus in the Order Dothideales (Ascomycota) and probably the largest genus of plant pathogens in the fungal kingdom. Together with the associated asexual stages, this genus contains (at least) 3,000 species. Dothideales

constitute an extremely large and diverse order with more than 1,000 named species. The heterothallic ascomycetous fungus *M. graminicola* (Fuckel) J. Schroeter in Cohn is the sexual state of *Septoria tritici* Roberge in Desmaz., and the causal agent of septoria tritici leaf blotch of wheat. The fungus is a non-appressorial dimorphic pathogen and transition from a yeast-like to a filamentous growth phase is required for successful infection. It is a hemibiotroph with a biotrophic phase of about ten days followed by a rapid shift to a necrotrophic phase resulting in the development of irregular chlorotic lesions that rapidly develop into necrotic blotches suggesting an active role of phytotoxic compounds (Kema et al., 1996b; Mehrabi et al., 2006). Anamorphic asexual pycnidia containing pycnidiospores and teleomorphic sexual pseudothecia containing ascospores can be formed in the necrotic lesions. Sexual reproduction of *M. graminicola* can be completed within five weeks of infection (Hunter et al., 1999) and causes high genetic diversity in natural populations (Chen and McDonald, 1996). A highly diverse pathogen population may easily adapt to selection exerted by changing conditions such as the use of fungicides or new (resistant) wheat cultivars. As a result of gene-flow between geographically separated populations, the level and distribution of genetic variation among *M. graminicola* populations is high in the global population (McDonald et al., 1995). Due to its typical pathogenic behavior with an active sexual cycle in nature, *M. graminicola* is adopted as a model fungus of the Order Dothideales in studies on pathogenicity factors and population genetics (Goodwin et al., 2004; Zhan et al., 2003). In *M. graminicola* research novel molecular and genetic tools have been exploited to increase our understanding of this pathosystem. The pathogen can be transformed and efficient gene disruption protocols have been implemented (Zwiers and De Waard, 2001). A detailed genetic linkage map, a database with more than 30,000 expressed sequence tags (ESTs), and the availability of several genomic libraries have facilitated sequencing the genome of *M. graminicola* that is recently released (Kema et al., 2002, 1996a, 2003). The availability of the genome sequence will facilitate functional analysis of genes and proteins required for virulence and will be invaluable for development of novel strategies to control this devastating fungal pathogen in wheat fields around the world.

Disease management

Disease management of septoria tritici blotch relies on breeding for resistance, cultural practices, and chemical control. As *M. graminicola* produces air-borne sexual ascospores that migrate over long distances, random mating (sexual) reproduction can lead to a global population with a high level of genetic diversity, within the pathogen population which can easily adapt to resistant cultivars by changing their virulence spectrum, leading to a gene-for-gene relationship (Brading et al., 2002; Chen and McDonald, 1996). Cultural practices aimed to reduce the amount of inoculum available for infections and to reduce the exposure time of the crop to the pathogen constitute the main strategies of septoria disease control, particularly in low input crop production systems (Bannon and Cooke, 1998). In wheat cultivating countries where high yields and disease incidence justify additional measures, different classes of fungicides have been used for control of the disease.

Chemical control

M. graminicola on wheat can be controlled by various protective fungicides such as the dithiocarbamates maneb, mancozeb, and zineb and the aromatic fungicide chlorothalonil (Eyal and Wahl, 1975; Hims and Cook, 1992), but they have to be applied repeatedly during the growing season, which significantly increases the cost of disease management. Hence, after their introduction in the 1970s, systemic benzimidazole fungicides such as benomyl with curative activity and long protective action were extensively used (Sanderson and Gaunt, 1980). The evolution of benzimidazole-resistant strains reduced or even completely abandoned the use of these fungicides (Fisher and Griffin, 1984) and from the 1980s onwards sterol demethylation inhibitors (DMIs) were extensively applied such as the azole fungicides cyproconazole, epoxiconazole, propiconazole, tebuconazole, and triadimefon. Most azole fungicides are systemic and possess both protective and curative disease control activity (Kuck and Scheinpflug, 1986). Azole fungicides have a site-specific mode of action inhibiting the

enzyme sterol 14α-demethylase involved in sterol biosynthesis (Sisler and Ragsdale, 1984). For that reason, plant pathogens have a resistance risk to azole fungicides. Azole resistance indeed emerged in practice but developed relatively slowly as compared to other classes of fungicides and the degree of resistance may be relatively low. As a consequence, modern azoles with high efficacy are still widely used in disease control (De Waard, 1994; Schepers, 1985).

The discovery of a new class of broad spectrum systemic fungicides, the strobilurins, was inspired by the discovery of a group of natural fungicidal derivatives of ß–methoxyacrylic acid, the simplest of which are strobilurin A, oudemansin A (from the basidiomycetous wood-rotting fungi *Strobilurus tenacellus* and *Oudemansiella mucida*, respectively) and myxothiazol A (from the bacterium *Myxococcus fulvus*). Strobilurins were introduced to the market in 1996, and within just four years the market share of these fungicides expanded to more than 10% of the total global fungicide market. Some strobilurin fungicides efficiently control *M. graminicola* after protective and curative applications (Bartlett et al., 2002). The strobilurins are also known as quinone outside inhibitor (QoI) fungicides or QoIs, since their mode of action is based on inhibition of mitochondrial respiration by binding to the ubiquinol oxidation centre (Qo site) of cytochrome b, and thereby blocking electron transfer between this mitochondrial enzyme and cytochrome c_1 in the cytochrome bc_1 protein complex (Complex III) located in the inner mitochondrial membrane. As strobilurin fungicides are single site inhibitors, a single point mutation in the cytochrome b gene can strongly reduce affinity of the encoded enzyme for strobilurins and cause fungicide resistance (Gisi et al., 2000).

Strobilurin resistance was first reported in the wheat powdery mildew pathogen, *Blumeria graminis* f. sp. *tritici* (Felsenstein, 1999). Resistance occurred worldwide soon after the first applications of these fungicides and is based on a specific point mutation in cytochrome b (the substitution of glycine by alanine at position 143; G143A) (Sierotzki et al., 2000b). A similar substitution was identified in strobilurin-resistant strains of *Pseudoperonospora cubensis* on cucumber (Ishii et al., 2001), *Plasmopara viticola* on grape (Heaney et al., 2000), *Venturia inaequalis* on apple (Steinfeld et al., 2001), and *Mycosphaerella fijiensis* on banana (Sierotzki et al., 2000a). In 2003, the Fungicide Resistance Action Committee (FRAC http://www.frac.info/) reported

widespread strobilurin resistance in *M. graminicola* in all major wheat growing areas of Western Europe. An important reason for the fast resistance development is the high selection pressure imposed on the fungal population by extensive and frequent applications of strobilurins in wheat fields (Leroux et al., 2005). Strobilurin resistance in *M. graminicola* is also ascribed to the G143A substitution in cytochrome b (Fraaije et al., 2005; Fraaije et al., 2003; Ware et al., 2006). However, the possibility exists that additional resistance mechanisms play a role as well (Gisi et al., 2002), either alone or in combination with the G143A substitution. Potential additional resistance mechanisms that may operate include (i) reduced uptake or increased efflux, which reduces the intracellular concentration of the fungicide, (ii) overexpression of the target site that results in a titration effect of the compound, (iii) compensation of the toxic effects through alternative biosynthetic pathways, (iv) detoxification, and (v) lack of conversion to active compound (Steffens et al., 1996). Our previous studies demonstrated that drug efflux transporters play a role in sensitivity to azole and strobilurin fungicides in *M. graminicola* and it is possible that this mechanism operates alone or in combination with resistance based on the G143A substitution in cytochrome b of the pathogen (Roohparvar et al., 2006b; Zwiers et al., 2002).

Drug transporters

Survival and reproduction of microorganisms can depend on their ability to produce metabolites toxic to competing organisms and to resist the deleterious effects of such compounds. In addition, chemical control of plant diseases and mammalian mycosis has imposed selection pressure on fungal pathogens leading to development of various resistance mechanisms against different synthetic antifungal agents such as fungicides, antimycotics and drugs. Mechanisms of insensitivity or resistance to fungitoxic compounds can relate to qualitative factors such as the absence or presence of a sensitive target site or to quantitative factors such as uptake, transport, storage and metabolism. Drug transporters represent quantitative factors that can provide protection of organisms against natural toxic products and fungicides. In plant pathogens, drug

transporters function in baseline sensitivity to fungicides, multidrug resistance (MDR) and virulence on host plants. They can also actively secrete endogenous fungal toxins into the outer environment by which they prevent accumulation of these compounds in fungal cells and prevent or reduce suicidal toxicity.

Two important families of drug transporters that mediate membrane transport are the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) of transporters. The ABC and MFS superfamilies account for nearly half of the solute transporters encoded within the genomes of lower and higher eukaryotic organisms (Del Sorbo et al., 2000; Pao et al., 1998). Upon ATP hydrolysis, ABC transporters can transport a broad spectrum of cytotoxic, hydrophobic agents ranging from ions to macromolecules, against a concentration gradient and are characterized as primary active transporters (Bauer et al., 1999; Theodoulou, 2000). ABC transporters are determinants of azole sensitivity of laboratory strains of *M. graminicola* (Zwiers et al., 2002) and may account for variations in base line sensitivity of field strains of the pathogen to these fungicides (Stergiopoulos et al., 2003). MFS proteins are characterized as secondary transporters and constitute the largest protein family within all three domains of life (archaea, eubacteria and eukaryota). The role of MFS transporters in fungicide sensitivity and resistance, especially to strobilurins, was supported by the recent characterization of the *M. graminicola* MFS transporter MgMfs1 (Roohparvar et al., 2006a).

Modulators of drug transporters

The important role of ABC transporters in MDR of cancer cells has led to a wide interest in pharmacological agents (modulators) that inhibit the activity of these membrane proteins leading to accumulation of drugs and increase of their efficiency against cancer cells (Robert and Jarry, 2003). At present, MDR is a world-wide problem that includes failure of chemotherapy of cancer and control of bacterial and fungal diseases of humans, animals and plants. In oncology, modulators are classified as first generation modulators (compounds already used in clinics for other indications), second

generation modulators (analogues of these drugs), and third generation modulators, (drugs of original structure specifically developed for the purpose of MDR reversal) (Robert and Jarry, 2003). Many natural toxic compounds such as plant alkaloids and flavonoids have also been described as multidrug pump inhibitors (Gus et al., 2001; Lania-Pietrzak et al., 2005). For instance, isoflavones can act as potentiators of antibacterial activity of natural toxic plant compounds, indicating that they enhance the activity of (endogenous) natural antimicrobials (De Waard, 1997; Morel et al., 2003). Recently, the synthetic flavonoid derivative 4'-hydroxyflavone, was identified as an inhibitor of fungicide efflux in the plant pathogen *Pyrenophora tritici-repentis*, capable of restoring fungicidal activity against fungicide-resistant strains to normal wild-type sensitivity (Reimann and Deising, 2005). Medical modulators that synergize fungicide activity *in vitro* have been reported for *Aspergillus nidulans*, *Botrytis cinerea* and *M. graminicola* (De Waard and Van Nistelrooy, 1982; Hayashi et al., 2003; Stergiopoulos and De Waard, 2002).

Modulators of ABC transporter activity that potentiate the activity of azole fungicides towards plant pathogens *in vitro* may contribute to indirect disease control activity. This may be the case when ABC transporters, sensitive to modulators, function in secretion of virulence factors (*e.g.* phytotoxins) or in protection against antimicrobial plant compounds but hardly show any *in vitro* toxic activity. Such compounds may constitute a new class of modern antifungals.

Outline of the thesis

The aim of this thesis was to investigate the role of MFS and ABC drug efflux transporters of the wheat pathogen *M. graminicola* in fungicide sensitivity, multidrug resistance and pathogenicity, and to study the effects of modulators of drug transporters on the activity of azole fungicides against this pathogen both *in vitro* and *in planta*.

Chapter 1 gives an introduction on both the importance of wheat as a food crop and the septoria leaf blotch disease, caused by *M. graminicola.* Management and particular chemical control of the pathogen on wheat are discussed. Furthermore, a short

introduction on MFS and ABC drug transporters, their modulators and their significance in plant pathogenic fungi in general, and in *M. graminicola* in particular, is presented.

Chapter 2 reviews MFS transporters with emphasis on drug efflux families and their significance in plant pathogenic fungi. Classification, comparative analyses, physical characteristics, transport mechanisms, substrate ranges, and physiological functions of MFS transporters are included. The role of these proteins in secretion of various types of antifungal compounds (host-specific toxins, mycotoxins, antibiotics, plant defense compounds and fungicides), MDR and virulence of fungal plant pathogens are also reviewed.

Chapter 3 describes the functional characterization of the MFS transporter gene *MgMfs1*, the first MFS transporter gene cloned from *M. graminicola* with high homology to MFS transporters with the DHA14 configuration. The phenotypic characterization of *MgMfs1* disruption mutants demonstrates that MgMfs1 functions as a strong multidrug transporter. Results described in this chapter indicate that MgMfs1 is not involved in virulence on wheat. However, it facilitates the transport of fungal toxins, plant metabolites, and fungicides suggesting a role in fungicide sensitivity and MDR, particularly to strobilurin fungicides and fungal toxins.

Chapter 4 describes in more detail the significance of MgMfs1 in sensitivity of *M. graminicola* to the strobilurin fungicide trifloxystrobin. The transporter appears to function in strobilurin sensitivity of the pathogen *in vitro* as well as *in planta*. Furthermore, results indicate that increased efflux activity of the drug transporter may safeguard normal membrane function and fitness of strobilurin-resistant strains of *M. graminicola* with a high degree of resistance due to the G143A mutation in the target enzyme of the fungicide, cytochrome b.

Chapter 5 focuses on the identification and functional analysis of MgAtr7, a novel type of ABC transporter that seems to be unique for *M. graminicola* and *Fusarium graminearum*. The genomic sequence of *MgAtr7* shows high homology to other fungal ABC transporters involved in azole sensitivity and resistance. However, *MgAtr7* encodes a novel hybrid type of ABC transporter with the $[NBF\text{-}TM]_2$ configuration fused at the N- terminus to a protein domain characteristic for a dityrosine / pyoverdine biosynthesis protein (DIT1_PvcA). Functional analysis indicated that this gene has a

role in iron homeostasis. The transporter is neither involved in virulence nor in protection against fungicides or other xenobiotics.

Chapter 6 describes the first steps in the discovery and development of modulators of ABC transporter activity that potentiate the activity of azole fungicides towards plant pathogens and may possess indirect antifungal activity against *M. graminicola on planta* but not *in vitro*. These results indicate that these compounds indeed may act as disease control agents and can be exploited in the development of novel commercial modulators.

Chapter 7 gives a general discussion with a broader perspective on the most important results presented in this thesis such as the capacity of the MFS transporter, MgMfs1, to transport antifungal compounds and the impact of this finding on fungicide sensitivity and virulence. Attention is especially focused on the role of the transporter in sensitivity and resistance to strobilurins, a relatively new class of fungicides. The potential role of ABC transporter MgAtr7 in iron metabolism and siderophore production is discussed. Modulators of activity of drug transporters are discussed as lead products for a new generation of disease control agents. Some additional unpublished data are also included in this chapter.

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Chapter 2

Major facilitator superfamily (MFS) transporters and their significance in plant pathogenic fungi

(To be submitted in modified form)

Abstract

The major facilitator superfamily (MFS) transporters are secondary membrane transporters that constitute the largest protein family within all kingdoms of life. MFS transporters are involved in uniport, symport, or antiport of various compounds over membranes. In fungal populations resistant to antimycotics, drugs or fungicides it has been shown that overexpression of MFS drug efflux transporters can provide protection against these compounds and, hence, eventually may lead to multidrug resistance (MDR), a serious threat to the successful control of fungi pathogenic on different types of organisms. In fungal plant pathogens, these transporters may also function in baseline sensitivity to fungicides and in virulence on host plants by providing protection against toxic plant metabolites and by secretion of fungal pathogenicity factors, respectively. This review focuses on MFS transporters of fungi with respect to their classification, physical characteristics, substrate range, mechanism of transport, and their function in protection against antifungal compounds. The review specifically pays attention to members of this transporter family identified in different plant pathogenic fungi and their function in pathogenesis. The impact of fungal MFS drug transporters on fungicide baseline sensitivity, MDR, and drug discovery is also discussed.

Keywords: MFS transporter; Drug efflux pump; Host-specific toxin; Mycotoxin; Pathogenicity factor; Pathogenesis; Plant defense; Multidrug resistance; Fungicide; *Aspergillus* spp.; *Botrytis cinerea*; *Cercospora kikuchii*; *Cochliobolus carbonum*; *Dothistroma pini*; *Fusarium sporotrichioides*; *Gibberella* spp.; *Saccharomyces cerevisiae*

Introduction

In natural environments, successful survival and reproduction of microorganisms strongly depend on their ability to secrete metabolites toxic to competing organisms and to counteract the effects of toxic compounds of either endogenous or exogenous origin. Plant-pathogenic fungi may need to secrete host-specific toxins, host-non-specific mycotoxins or other virulence factors to establish a compatible relationship with their hosts. On the other hand, they must be able to resist the toxic effects of antimicrobial plant compounds such as phytoalexins and phytoanticipins. In addition, chemical control of fungal plant diseases and mammalian mycosis has imposed selection pressure on fungal pathogens leading to development of various resistance mechanisms against synthetic antifungal agents such as fungicides, antimycotics and drugs. Different qualitative and quantitative mechanisms are involved in fungal insensitivity or resistance against toxic compounds. Qualitative mechanisms relate to the presence or absence of a sensitive target site, whereas quantitative factors may relate to differential affinity of the target site between organisms and the effective toxic concentration built up at the target site as a consequence of uptake, transport, storage, and metabolism (De Waard et al., 2006; Del Sorbo et al., 2000; Stergiopoulos et al., 2002).

By directly influencing the effective concentration at target sites, membrane efflux systems act as crucial quantitative factors involved in sensitivity to natural toxic compounds and fungicides. Moreover, transporters play vital roles in cellular metabolism and activities by mediating uptake of essential nutrients, maintenance of ion homeostasis, secretion of end products of metabolism, environmental sensing and cell communication, and other important cellular functions (Andrade et al., 1999; Balzi and Goffeau, 1995; Higgins, 1992).

The importance of transport processes is reflected by the fact that over 400 families of membrane-bound transporters have been identified and classified (Saier et al., 2006). Two important superfamilies are the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) of transporters. The ABC and MFS superfamilies account for nearly half of the transporters encoded within the genomes of microorganisms. They are also prevalent in higher organisms (Del Sorbo et al., 2000; Higgins, 1992; Pao et al., 1998). ABC transporters can transport a broad spectrum of cytotoxic, hydrophobic

agents ranging from ions to macromolecules, against a concentration gradient using the energy derived from ATP hydrolysis, and therefore are primary active transporters (Bauer et al., 1999; Theodoulou, 2000). MFS transporters are proteins that constitute the largest protein family within all three domains of life (archaea, eubacteria and eukaryota). This superfamily of secondary transporters comprises over 500 described proteins involved in the uniport, symport, or antiport of various compounds over chemiosmotic ion gradients. Proteins that function as uniporters generally mediate the uncoupled transport of a single substrate across the membrane. Those that function as symporters mediate the coupled transport of two substrates in the same direction, whereas antiporters have the ability to transport two substrates in the opposite direction (Paulsen et al., 1996; Saier et al., 1999). Functions of ABC transporters are being studied extensively in nearly all types of prokaryotic and eukaryotic organisms (Ernst et al., 2005; Jungwirth and Kuchler, 2006; Lage, 2003; Prasad et al., 2006, 2002). ABC transporters of plant pathogenic fungi have been extensively reviewed for their role in efflux of biotic and xenobiotic toxic compounds and their impact on fungicide sensitivity, multidrug resistance (MDR) and virulence (De Waard, 1997; De Waard et al., 2006; Del Sorbo et al., 2000; Levy, 1992; Stergiopoulos, 2003). MFS transporters and their roles in cell life have also been studied in many organisms (Goffeau et al., 1997; Lewinson et al., 2006; Pao et al., 1998; Prasad et al., 2006) with the exception of fungal plant pathogens. In this review we present an overview of MFS transporters with emphasis on drug efflux families and their significance in plant pathogenic fungi. We will discuss the role of these transporters in secretion of host-specific toxins, other mycotoxins, and virulence factors, in protection against antibiotics, antimicrobial plant metabolites, fungicides, and in the development of MDR.

Classification of MFS transporters

Various transport systems differ with respect to their predicted membrane topology, energy coupling mechanism, substrate specificity and function (Saier, 2000). The high diversity within transporter protein families in general and the major facilitator superfamily in particular has indicated the necessity to classify these proteins into

phylogenetic families. Classification systems are valuable guides for scientists interested in structural, functional and evolutionary aspects of transporter families. Using transport classification systems, the significance of each family in cell physiology may be recognized. Ultimately, biochemical, molecular genetic and biophysical characteristics, and functions of all members can be predicted, comprehensive mechanistic models can be developed, and the evolution of transporters can be understood. The classification of transporters was initially based on comparative amino acid sequence analysis of various transport proteins, and enabled the identification of a number of distinct families and superfamilies (Griffith et al., 1992; Saier, 1994). Within the MFS transporters, Marger and Saier (1993) identified five distinct clusters or families of membrane transport proteins involved in drug resistance, sugar uptake, uptake of Krebs-cycle intermediates, phosphate ester/phosphate antiport, and oligosaccharide uptake. A computer-aided (*in silico*) analysis of all 5,885 predicted open reading frames of the eukaryotic model system *Saccharomyces cerevisiae* identified 186 potential MFS proteins, of which 149 were clustered into 23 families. Putative permease functions were assigned to 12 families, including families involved in sugar, amino acid, and multidrug transport (Nelissen et al., 1997). Pao et al. (1998) constructed phylogenetic trees exclusively based on sequence similarity and divided all the recognized members of the MFS into 17 families. Interestingly, they found a clear correlation between the assigned phylogenetic family and its function. Each of the families seemingly recognizes and transports a distinct class of structurally related compounds. Hence, a classification system was made based on both phylogeny and function, with the potential to include all known permeases with available sequence data and the flexibility to accommodate permeases derived from future genome sequences and biochemical analyses (Saier, 1998). Following studies expanded the MFS to 29 established and five putative families, and described structural, functional, and mechanistic features of the constituent permeases (Saier et al., 1999).

A systematic approach to classify transport systems is represented in a web resource (http://www.tcdb.org/) as the Transporter Classification Database (TCDB) which includes a compilation of published information from over 10,000 references (Saier et al., 2006). The transporter classification (TC) system parallels but differs from the strictly functional Enzyme Commission (EC) system of the International Union of

Biochemistry and Molecular Biology (IUBMB) for classification of enzymes. The basis of classification in the TC system relies primarily on the mode of transport and energy coupling mechanism and secondly on molecular phylogeny and substrate specificity (Saier, 1999, 2000). A combination of four numbers and a letter within the TC number are used to assign each individual transporter. Each component corresponds to one of the five criteria used for a particular type of transporter. Therefore, a TC number is described with the five components D1.L1.D2.D3.D4. D1 (a single number) represents the transporter class, L1 (a single letter) the transporter subclass, which, for example in case of primary active transporters, refers to the energy source used to drive transport. These two components constitute the classes and subclasses as follows: class 1. channels / pores containing five subclasses of 1.A. α-type channels, 1.B. β-barrel porins, 1.C. pore-forming toxins (proteins and peptides), 1.D. non-ribosomally synthesized channels, 1.E. holins; class 2. electrochemical potential-driven transporters containing three subclasses of 2.A. porters (uniporters, symporters, antiporters), 2.B. non-ribosomally synthesized porters, 2.C. ion-gradient-driven energizers; class 3. primary active transporters containing five subclasses of 3.A. P-P-bond-hydrolysis-driven transporters, 3.B. decarboxylation-driven transporters, 3.C. methyltransfer-driven transporters, 3.D. oxidoreduction-driven transporters, 3.E. light absorption-driven transporters; class 4. group translocators containing two classes of 4.A. phosphotransfer-driven group translocators, 4.B. the nicotinamide ribonucleoside (NR) uptake permease (PnuC) family; class 5. electron transport carriers containing two subclasses of 5.A. transmembrane 2-electron transfer carriers, 5.B. transmembrane 1-electron transfer carriers; and the two reserved classification categories, class 8. accessory factors involved in transport containing subclass of 8.A. auxiliary transport proteins; class 9. incompletely characterized transport systems containing three subclasses of 9.A. recognized transporters of unknown biochemical mechanism, 9.B. putative uncharacterized transport proteins, and 9.C. functionally characterized transporters lacking identified sequences. TC classes 6 and 7 are currently unused but will be introduced if additional classes of transporters are discovered. D2 (a number) corresponds to the transporter family (or sometimes a superfamily); D3 (a number) corresponds to the subfamily (or the family of a superfamily) in which a transporter is found; and D4 (a number) corresponds to the transporter itself. This refers to a specific

transport system with a defined range of substrates, a known polarity of transport, an energy source that drives conducted movement of the substrate and a mechanism of action. If multiple dissimilar subunits are present, they are numbered S1, S2, S3...Sn (Busch and Saier, 2002, 2004; Saier, 2000). According to this system, the MFS multidrug efflux transporter CaMDR1 from Candida albicans is indicated under a TC number of 2.A.1.2.6 which refers to class 2 comprising the electrochemical potential-driven transporters, subclass 2.A, the porters (uniporters, symporters, antiporters), (super)family 2.A.1, the major facilitator superfamily (MFS), (sub)family 2.A.1.2, the drug:H^+ antiporter-1 (12 spanner) (DHA1) family, and substrate range of 2.A.1.2.6, (benomyl, cycloheximide, methotrexate, fluconazole, etc.):H^+ antiporter. Based on the TC system, approximately 3,000 distinct proteins from all kinds of known organisms have been organized into more than 400 transporter families (Saier et al., 2006). The MFS and ABC superfamilies alone consist over 60 and 50 families of transport proteins, respectively. The ubiquitous MFS families of DHA1 (12 spanner) and DHA2 (14 spanner) which are known to function in drug efflux in prokaryotes and eukaryotes, are indicated under TC numbers of 2.A.1.2 and 2.A.1.3, respectively.

Comparative analyses

Over the last 25 years, the fast growing number of sequenced genomes and a wealth of biochemical and molecular genetic studies have led to identification of an enormous number of primary and secondary transporters from prokaryotic and eukaryotic organisms (Ren and Paulsen, 2005). Without these recent advances analyses of membrane transporters would be limited to the examination of individual transporter genes of a few organisms. The progresses in whole-genome sequencing made it possible to compare transport and other essential cellular processes across a range of organisms. The genomes of over 450 prokaryotes and eukaryotes including 13 fungi have now been completely sequenced. Furthermore, over 1,700 additional genome sequencing projects, including nearly 160 fungi, are currently underway (Gold Genomes Online Database, http://www.genomesonline.org/) (Bernal et al., 2001; Janssen et al., 2005). Recently, a total of 40,678 predicted transport proteins from 141 organisms (whose complete

genome sequences are available), including 115 Eubacteria, 17 Archaea, and nine Eukaryota (three fungi), were classified into 134 families, including seven families of primary transporters, 80 families of secondary transporters, 32 channel protein families, two phosphotransferase systems (PTSs), and 13 unclassified families (Ren and Paulsen, 2005). Comparative analysis of these membrane transport systems shows a wide range of variation among prokaryotes and eukaryotes in the relative usage of energy coupling mechanisms used to drive transport processes. As mentioned earlier, primary and secondary carriers are ubiquitously present in all organisms but their numbers and ratios may differ significantly. In prokaryotic and unicellular eukaryotic systems, primary and secondary carriers are the predominant types of transporters, together constituting more than 90% of all transporters. In higher eukaryotes, channel proteins occur more frequently than in prokaryotes constituting 12 and 43% in plants and primates, respectively (Ren and Paulsen, 2005). Compared to eukaryotes, prokaryotic organisms rely heavily on primary active transporter systems, mainly because of the usage of ABC uptake systems that are absent in eukaryotes (Dean and Allikmets, 2001). In fungi, secondary transporters (76–80%) are overrepresented mainly because of the gene expansion of two types of functionally diverse MFS transporters; drug efflux pumps and sugar symporters (Borkovich et al., 2004; Galagan et al., 2003). In general, organisms with a large genome size possess a relatively high number of transporters. In prokaryotes and unicellular eukaryotes, this increase of the number of transporters with genome size is primarily due to increased diversity of transporter types. In contrast, in multicellular eukaryotes, this increase is mainly due to the higher number of paralogs originating from gene duplication or expansion in specific transporter families.

Physical characteristics and sequence homology within MFS families

MFS transporters are located in the plasma membrane or in membranes of intracellular compartments, such as vacuoles, endoplasmatic reticulum, peroxisomes and mitochondria. Typically, the structural unit of these transporters is composed of two homologous halves each containing six transmembrane spanning α-helical segments (the so called transmembrane domain (TMD) connected by a large cytoplasmic loop

between TMDs 6 and 7. They typically consist of 12 or 14 transmembrane domains with a molecular mass ranging between 45 and 90 KDa. The structure of the two halves of MFS transporters with 12 TMDs is similar, suggesting that a gene duplication event may have occurred from an ancestral gene encoding a six TMD protein, while the transporters with 14 TMDs configuration may have emerged by acquisition of two additional TMDs at the C-terminus. (Pao et al., 1998; Paulsen and Skurray, 1993; Rubin et al., 1990; Stergiopoulos et al., 2002). The two drug:H^+ antiporter (DHA) families of *S. cerevisiae*, DHA12 and DHA14, are predicted to contain 12 and 14 transmembrane spanners, respectively (Pao et al., 1998; Sá-Correia and Tenreiro, 2002).

Unlike ABC transporters, MFS transporters have no well defined characteristic signature. However, a 13-residue amino acid consensus motif present between TMD-2 and TMD-3 has been identified (Pao et al., 1998). Moreover, significant sequence homology occurs between all members of the same family (Pao et al., 1998). In MFS proteins with either 12 or 14 TMDs motifs have been identified that are characteristic for each group or are conserved in both groups. The conservation of these motifs suggests that they are essential for the function of these proteins (Paulsen and Skurray, 1993). Sequence similarity between MFS families is substantially greater in their N-terminal than in their C-terminal halves (Griffith et al., 1992; Marger and Saier, 1993).

Substrate range, substrate binding and mechanism of transport

MFS proteins are involved in transport of various substrates including amino acids, drugs, iron-siderophores, Krebs-cycle metabolites, nucleosides, neurotransmitters, osmolites, peptides, phosphorylated glycolytic intermediates, organic and inorganic anions and cations, polyols, sugars, and vitamins. Unlike ABC proteins, MFS transporters only mediate transport of relatively small-sized molecules (Pao et al., 1998; Stergiopoulos et al., 2002). In some Gram-positive bacteria, efflux is exclusively mediated by MFS pump proteins such as NorA or QacA in *Staphylococcus aureus* and PmrA in *Streptococcus pneumoniae* (Cattoir, 2004). Most efflux systems typically transport a narrow range of structurally related substrates e.g. an antibiotic exporter of bacteria capable of extruding a specific antibiotic and some structural closely related

analogs (Levy, 1992). However, export systems capable of transporting a wide range of structurally unrelated compounds such as multidrug transporters have also been identified (Lewis, 1994). For MFS transporters with a wide substrate range, data suggest that the C-terminal region primarily determines substrate specificity whereas the N-terminal region is involved in the activation and energization of transport (Griffith et al., 1992; Paulsen et al., 1996). Increasing evidence suggests the presence of more than one drug-interaction site in secondary multidrug transporters. For the proton motive force-dependent MFS transporter LmrP from *Lactococcus lactis*, it has been suggested that some drugs inhibit the LmrP-mediated transport of Hoechst 33342 through competition for the same drug-binding site, whereas others through non-competitive binding to a different site (Putman et al., 1999). Several families of proton motive force (PMF)-dependent multidrug efflux proteins with broad substrate specificity but different evolutionary origin exist. Although the molecular basis of the broad substrate specificities of these MFS transporters is not well understood, some conclusions can be drawn based on various studies. Apparently, the physical characteristics of compounds, such as charge, hydrophobicity or amphipathicity are more important than their chemical structures in determining specificity. The same seems to hold true for the substrates of ATP-dependent multidrug efflux pumps. These findings suggest that some PMF-dependent efflux systems may have a similar mechanistic basis to recognize structurally unrelated drugs as ABC transporters (Lomovskaya and Lewis, 1992; Neyfakh et al., 1993; Paulsen et al., 1996). The phylogenetic trees based on genome-sequence analyses of five hemiascomycetous yeast species clearly demonstrate that a similar set of gene (sub)families of MDR proteins of MFS and ABC transporters were already present in the common ancestor of all fungal species. However, striking differences exist between the two superfamilies with respect to the evolution of the various subfamilies. Within the ABC superfamily all six half-size transporters with six TMDs and most full-size transporters with 12 TMDs are encoded by a single gene. Gene duplication in genomes is not observed. Among the MFS transporters, the DHA2 family (TC 2.A.1.3) is more diverse between species than the DHA1 family (TC 2.A.1.2).

MFS proteins are secondary active transporters. The energy needed for transport is provided by the electrochemical potentials of the transported substrates (proton motive

force) across membranes (Lewis, 1994; Marger and Saier, 1993; Pao et al., 1998; Paulsen et al., 1996). Although, it is far from clear how MFS transporters are able to transport such a diverse range of compounds with different structures, the transport mechanisms may be all fundamentally similar (Marger and Saier, 1993). In the bacterial tetracycline/H^+ antiporter TetA(B), used as a model for DHA1 MFS transporters, the presence of a central water-filled channel with a thin permeability barrier at the middle of the channel has been demonstrated. The distances between transmembrane helices across the channel are increased at the periplasmic side when tetracycline binds to TetA(B), and subsequently the channel acts as at least a part of the tetracycline translocation pathway. Metal-tetracycline complex enters into TetA(B) through the gate composed of loop 2–3 and loop 4–5. After transport through the water-filled channel, it is released through an exit gate. The proton translocation seems to be located in the hydrophobic core composed of helices 7, 8 and 9 and, thus, is separated from the substrate translocation and the water-filled channel. This remote-conformational coupling between proton translocation and substrate transport may be a general rule for secondary transporters (Tamura et al., 2003).

MFS transporters functioning in protection against antifungals

A consequence of the broad substrate specificity of MFS transporters is their involvement in a broad range of cell functions such as uptake of nutrients, secretion of cell cycle metabolites, protection against toxic compounds, and maintenance of an electrochemical gradient. This is also true for filamentous fungi and yeasts where they play also a role in sporulation, cell to cell communication and pathogenesis (Pao et al., 1998). Transporters contribute to the genetic diversity of fungal populations by secretion of mating type factors during sexual reproduction (Christensen et al., 1997; Kuchler et al., 1989).

As MFS transporters are able to secrete a wide variety of natural and synthetic toxic compounds of endogenous or exogenous origin into the environment (Stergiopoulos et al., 2002), they avoid or lower the toxic concentrations of these compounds at target sites by preventing or reducing their accumulation inside the cells (Nelissen et al., 1997;

Pao et al., 1998). (Over)expression of DHA12 and DHA14 MFS transporters can mediate the development of MDR to unrelated antifungals (De Waard and Van Nistelrooy, 1979, 1980; Prasad et al., 2006; Prasad and Kapoor, 2005). MDR is defined as the simultaneous resistance of organisms to unrelated compounds conferred by a single gene and its encoded protein. Overexpression of transport systems leading to MDR was first described for ABC transporters in human tumor cells resistant to anticancer drugs (Gottesman and Pastan, 1993). At present, MDR is also a serious threat to the effectiveness of medical drugs and agricultural fungicides (De Waard et al., 2006, 1995; Gottesman and Pastan, 1988, 1993; Prasad and Kapoor, 2005).

The development of fungal populations resistant to antimycotics, drugs or fungicides as a result of the widespread use of antifungal compounds has led to intensive studies of resistance mechanisms. These investigations demonstrated that besides ABC transporters, MFS transporters also function in protection against synthetic toxic compounds, such as fungicides, antimycotics and other non-related compounds and, hence, can be regarded as MDR agents. This phenomenon has been observed for various fungi with medical, microbiological or agricultural importance. In the genome of *S. cerevisiae* a role in MDR has been established for some of the (at least) 28 identified multidrug MFS transporters (Nelissen et al., 1997; Tenreiro et al., 2002, 2005; Vargas et al., 2004). For most of the others, a functional characterization is still missing (Sá-Correia and Tenreiro, 2002). In the opportunistic human pathogen *Candida albicans*, the MFS pump *CaMDR1* plays a key role in azole resistance as suggested by its high level of expression in azole-resistant clinical isolates (Prasad and Kapoor, 2005).

These transporters provide protection against toxic compounds present in their natural environment or avoid cytoplasmic accumulation of toxic secondary metabolites produced by the fungus itself. De Waard (1997) postulated that in plant pathogens, drug transporters of the ABC and MFS superfamilies can function in production and secretion of endogenous fungal pathogenicity factors (*e.g.* host specific-toxins or mycotoxins) and in protection against exogenous antimicrobial plant metabolites (*e.g.* phytoalexins), and therefore, can play a role in virulence. These postulations proved to be valid to both superfamilies of transporters since in recent years ABC and MFS

transporters of phytopathogenic fungi have been described as virulence or pathogenicity factors in pathogenesis on host plants (Stergiopoulos et al., 2003).

MFS transporters from plant pathogenic fungi

Aspergillus spp.

Aflatoxins are strong hepatocarcinogens found in contaminated food and feed and mostly produced by *A. flavus* strains (Kurtzman et al., 1986). Since pathway-specific mycotoxin transporters are often present in biosynthetic gene clusters, the clustering of aflatoxin biosynthetic genes has been studied in *A. parasiticus* and *A. flavus* (Trail et al., 1995; Yu et al., 2004). *aflT* is a MFS transporter gene from *A. parasiticus* located in the aflatoxin gene cluster between the polyketide synthase gene pksA and the P450-encoding cypA gene. The gene is also found at the same relative position in the genomes of *A. flavus* isolates. The predicted protein, AFLT, contains 14 TMDs and displays a relatively high degree of amino acid identity to other MFS transporters such as *Botryotinia fuckeliana* Bcmfs1 (Hayashi et al., 2002), *Cochliobolus carbonum* TOXA (Pitkin et al., 1996), *A. terreus* ORF10 (Kennedy et al., 1999), *Penicillium citrinum* MlcE (Abe et al., 2002), *Cercospora kikuchii* CFP (Callahan et al., 1999), and *Dothistroma pini* DotC (Bradshaw et al., 2002). However, *aflT* shows no similarity to TRI12 of *Fusarium sporotrichioides* (Alexander et al., 1999) and is not required for aflatoxin secretion (Chang et al., 2004).

Botrytis cinerea

Bcmfs1 is the first cloned and functionally analyzed MFS transporter gene from *B. cinerea*. The predicted protein, Bcmfs1 consists of 14 TMDs, and is the first described fungal MFS transporter involved in MDR capable of transporting both natural toxic compounds and fungicides. However, *Bcmfs1* replacement mutants of *B. cinerea* were not impaired in virulence on detached tomato leaves but did show an increased sensitivity to the natural toxic compounds camptothecin and cercosporin, produced by the plant *Camptotheca acuminata* and the plant pathogenic fungus *C. kikuchii*, respectively. Detailed analysis of Bcmfs1 overexpression mutants and a double

replacement mutant of *Bcmfs1* and the ABC transporter gene *BcatrD* indicated that Bcmfs1 also plays a role in the secretion of azole fungicides. A clear correlation was observed between azole sensitivity, *Bcmfs1* expression levels and fungicide accumulation levels. Therefore, Bcmfs1 may be involved in protection of *B. cinerea* against antimicrobial secondary plant metabolites accumulating during infection of host plants and against fungitoxic antimicrobial metabolites encountered in the environment during its saprophytic phase (Hayashi et al., 2002).

Cercospora kikuchii

Like many species belonging to the genus *Cercospora,* the soybean pathogen *C. kikuchii,* produces the phytotoxic polyketide cercosporin. The MFS transporter gene *CFP* (cercosporin facilitator protein) was identified as one of several cDNAs accumulating in the light, a condition known to stimulate cercosporin production. CFP is a 14 TMD-MFS transporter protein, able to secrete cercosporin and to contribute to resistance and self-protection of *Cercopora* spp. to this compound. Although multiple mechanisms are involved in cercosporin tolerance, it is at least partly provided by CFP. Disruption mutants of *CFP* are more sensitive to exogenously applied cercosporin and display a strong reduction in cercosporin production and virulence on soybean. Complementation of *CFP* disruption mutants with a functional *CFP* copy restores wild-type virulence on soybean (Callahan et al., 1999). Overexpression of *CFP* in *C. kikuchii* upregulates the production and secretion of cercosporin. Thus, CFP may also have a significant role in regulating toxin production (Upchurch et al., 2001). In order to assess the transport capacity of CFP and its contribution to cercosporin tolerance in more detail, *CFP* was expressed in the cercosporin-non-producing and cercosporin-sensitive fungus *Cochliobolus heterostrophus*. This resulted in increased tolerance to cercosporin due to secretion of the compound by CFP (Upchurch et al., 2002). Transgenic tobacco plants expressing *CFP* of *C. kikuchii* displayed smaller (but similar in number) frog-eye leaf lesions after inoculation with *C. nicotianae*. Accumulation of CFP in the leaf membrane fraction of CFP^+ transgenic plants was associated with decreased cercosporin phytotoxicity. Transgenic expression of CFP may have relevance for a disease control strategy in *Cercospora*-plant pathosystems where cercosporin is implicated in pathogen virulence (Upchurch et al., 2005).

Cochliobolus carbonum

The MFS transporter-encoding gene, *TOXA* from the maize pathogen *C. carbonum* is the first described MFS transporter involved in secretion of host-specific mycotoxins (Pitkin et al., 1996). TOXA is a MFS transporter with 10–13 predicted TMDs, probably involved in secretion of the host-specific cyclic tetrapeptide HC-toxin. Two linked copies of *TOXA* are present in the genome of HC toxin producing isolates of *C. carbonum.* The genes flank the *HTS1* gene that encodes the central enzyme in the HC-toxin biosynthesis gene cluster. *TOXA* genes have only been found in fungal strains that produce HC-toxin. Mutants with a single-disrupted copy of *TOXA* still produce the HC-toxin and are virulent on maize. As all attempts to obtain double knockouts for both copies of *TOXA* were unsuccessful, it has been suggested that the encoded proteins are involved in self-protection of *C. carbonum* against HC toxin and essential for survival of HC-toxin producing strains and their virulence on maize.

Interestingly, *Zm-mfs1*, a maize MFS transporter gene related to bacterial multidrug resistance efflux antiporters that export antimicrobial drugs and other compounds, has been identified as a maize defense-inducible gene. Expression of *Zm-mfs1* is induced by pathogens and defense-related conditions in various tissues involving both host and non-host interactions including *C. carbonum* and *C. heterostrophus* on maize. Zm-Mfs1 is hypothesized to be a prototype of a new class of plant defense-related proteins that could be involved in export of antimicrobial compounds produced by plant pathogens (Simmons et al., 2003).

Dothistroma pini

Dothistromin is a difuranoanthraquinone toxin with remarkable structural similarity to versicolorin B, a precursor of the aflatoxin family of compounds. The toxin is produced by *D. pini*, the causal agent of pine needle blight (Gibson, 1972) as well as by several *Cercospora* species (Stoessl and Stothers, 1985). Dothistromin is suggested to play a role in pathogenicity. Homologues of aflatoxin biosynthetic pathway genes have been identified in *D. pini*. The identified genomic *D. pini* clone contains four *dot* genes with similarity to genes in aflatoxin and sterigmatocystin gene clusters with predicted activities of a ketoreductase (*dotA*), oxidase (*dotB*), major facilitator superfamily

transporter (*dotC*), and thioesterase (*dotD*). It is speculated that the four *dot* genes, including the MFS transporter *dotC*, may comprise part of a dothistromin biosynthetic gene cluster (Bradshaw et al., 2002).

Fusarium sporotrichioides

Trichothecenes are fungal toxins commonly found in cereals and are mainly produced by *Fusarium* species. These mycotoxins have been associated with outbreaks of diseases both in humans and farm animals. Two well-known trichothecene mycotoxins are nivalenol (NIV) and deoxynivalenol (DON). Most of the genes involved in trichothecene biosynthesis are located within a gene cluster. In the maize pathogen *F. sporotrichioides*, this cluster includes the MFS transporter gene *Tri12*. The encoded protein TRI12 contains 14 predicted TMDs. Heterologous expression of this gene in *S. cerevisiae* results in a decreased sensitivity to trichothecene. *Tri12*-deletion mutants show a strong reduction in toxin production and an impaired growth *in vitro*. These findings indicate that TRI12 acts as a trichothecene transporter, is required for the secretion of the mycotoxins, and plays a role in self-protection of the pathogen against these endogenous mycotoxins (Alexander et al., 1999). The same seems to be true for the *F. graminearum* MFS transporter gene *Tri102*, a *Tri12* homologue, as it is located in the mycotoxin biosynthetic gene cluster and encodes a MFS trichothecene efflux pump (Wuchiyama et al., 2000).

***Gibberella* spp.**

The genes of the gibberellin (GA) biosynthesis pathway in *G. fujikuroi* are organized in a gene cluster of at least seven genes. The MFS transporter-encoding gene *smt* is located adjacent to the GA gene cluster. Functional analysis of *smt* indicated that the gene is expressed in mycelium grown under conditions stimulating GA production, but not under GA biosynthesis repressing conditions. However, *smt* replacement mutants did not show any reduction in GA yield and gibberellic acid or its precursors did not influence gene expression. Thus, despite its location adjacent to the GA gene cluster *smt* is not essential for the biosynthesis or secretion of GAs. However, it may play a role in sugar transport, since sugar alcohols, such as myo-inositol, sorbitol and mannitol, induce the expression of *smt* (Voss et al., 2001).

G. pulicaris (anamorph *Fusarium sambucinum*) is the causal agent of potato dry rot, and infects potato tubers where it is exposed to high amounts of antimicrobial compounds such as rishitin and lubimin. Transcription of *rin6* (Gpmfs-MDR1), a *G. pulicaris* gene encoding a MFS transporter is strongly induced upon mycelium treatment with rishitin suggesting a function in protection against rishitin (Weltring et al., 1998).

Practical implications and perspectives

Drug transporters as targets for new antifungals

The fact that MFS transporters of fungal plant pathogens can function as virulence factors and are involved in base-line sensitivity and MDR to fungicides, suggests that they might be interesting targets for disease control on host plants. Inhibition of MFS transporters may improve the efficacy of chemical control and reduce virulence of plant pathogenic fungi. Such inhibitors may lead to the discovery of new fungicides. Inhibitors of activity of ABC transporters are already described in the medical literature as chemosensitizers, blockers or modulators of ABC transporters and are tested in human chemotherapy as synergists of drugs or as compounds that annihilate MDR in cancer cells (Avendano and Menendez, 2002). These compounds can function as competitive or non-competive inhibitors of transport activity. Inhibitors of ABC transporter activity without cytotoxic action have been described for plant pathogenic fungi (De Waard and Van Nistelrooy, 1982; Hayashi et al., 2003; Roohparvar et al., 2006) and such compounds could be developed as synergists of agricultural fungicides or as compounds that prevent or reduce MDR in plant pathogens (De Waard et al., 2006). Combinations of azole or strobilurin fungicides with a hydroxyflavone derivative rendered fungicide-resistant *Pyrenophora tritici-repentis* isolates sensitive and prevented infection of wheat leaves (Reimann and Deising, 2005). Although, the development of modulators has been traditionally focused on ABC transporters and still needs to be validated in practice, the increasing evidence of the involvement of MFS transporters in fungicide sensitivity justifies a similar approach for MFS transporters.

Drug efflux mechanisms can interfere with screening programs aimed at the discovery of new fungitoxic compounds, since these transporters can reduce the activity of experimental fungicides. Fungal mutants that lack drug transporters may have a fungicide-hypersensitive phenotype. For that reason such mutants are useful as tester strains in fungicide discovery screening programs, since they may increase the chance for discovery of agricultural fungicides and antimycotics (Reimann and Deising, 2005; Skatrud, 2002). However, such compounds may have a risk for MDR development. To prevent this possibility, candidate compounds should not be substrates of MFS transporters. This can be avoided by further screening and optimization of compounds for similar fungitoxic activity to wild-type, disruption, and overexpression mutants of MFS transporters. Hence, deletion and overexpression mutants should also be included in the optimization of new fungicides.

Significance of overexpression mutants for increased production of antibiotics

Many fungal species are known to produce antibiotics and other secondary metabolites and their secretion might be mediated by efflux pumps such as MFS transporters. Traditionally, strain improvement aimed at increasing the levels of antibiotic production focuses on the increase of the metabolic flux. However, especially for metabolites which are toxic to the producer strain itself, it could be worthwhile to stimulate secretion in mutants overexpressing specific transporters.

Disease control agents

Modulators that inhibit the activity of MFS transporters with a role in fungal virulence can be developed as disease control agents if the transporters involved secrete antimicrobial plant metabolites. Inhibition of such transporters would enhance accumulation of these compounds in fungal cells and as a result utilize the natural defense strategies of host plants. Such compounds do not necessarily need to have *in vitro* fungitoxicity, and therefore, development of such compounds might result in the discovery of disease control agents without a direct fungitoxic activity. A prerequisite for the discovery of such compounds is their selective activity for the target organism.

Whole genome sequencing programs

The rapid increase in availability of whole-genome sequences of many fungi and fungus-like organisms and *in silico* comparative analyses have provided numerous novel fungal MFS genes. The function of transporter proteins encoded by these genes is only partly understood as the role of the majority of MFS transporters is based on predictions derived from sequence homologies and expression data. So, more functional analyses should be performed. Although a role of fungal MFS transporters in virulence and fungicide resistance has been validated, more research is needed to better understand these phenomena. This should be done in large-scale phenotyping of knock-out and overexpression mutants of the MFS transporter genes. In order to fully understand the mechanisms of transporter-mediated drug resistance, it is also important to understand the molecular mechanisms regulating the expression of the drug efflux pumps. In *S. cerevisiae* a number of transcription factors belonging to either the PDR (pleiotropic drug resistance) or YAP (yeast activator protein) regulatory network have been characterized. The PDR network in *S. cerevisiae* comprises a family of transcription regulators including *PDR1, PDR3, PDR7* and *PDR9* (Balzi and Goffeau, 1994, 1995). The involvement of *PDR1 and PDR3* in the regulation of the expression of the ABC drug efflux pump-encoding genes *PDR5, SNQ2* and *YOR1* is well-established (Balzi et al., 1994; Decottignies et al., 1995; Katzmann et al., 1994; Mahe et al., 1996). The hexose MFS transporters *HXT9* and *HXTll* involved in PDR of *S. cerevisiae* are also under the control of PDR1 and PDR3 (Nourani et al., 1997). Similar results have been found for the YAP network that includes eight alleles of the *YAP* family of regulators (*YAP1-8*) in *S. cerevisiae*. The two encoded proteins YAP1 and YAP2 are transcriptional activators involved in pleiotropic drug resistance (Fernandes et al., 1997). Overexpression of *YAP1* or *YAP2* leads to increased resistance to a variety of drugs and metals. Similar to PDR1 and PDR3, YAPl is also capable of regulating drug efflux pumps belonging to both ABC and MFS superfamilies (Alarco et al., 1997; Jungwirth et al., 2000). Regulatory systems that control expression of drug efflux pumps in yeasts and the remarkable number and functions of MFS transporters in biological systems are very complex. Therefore, more research is needed to get a better understanding of the regulation of transporter mediated resistance. Ultimately this might

lead to ways to directly interfere with the expression of MDR transporter genes, including regulatory genes, and, eventually the prevention of MDR.

Perspectives

Studies on fungal MFS transporters are exciting as they combine fundamental and applied aspects and may result in various practical applications. Advances in fungal whole-genome sequencing programs and molecular biological techniques, such as efficient fungal transformation and high-throughput functional analysis, will strongly stimulate this type of research in the near future and may lead to the discovery of modulators with new physiological functions and applications.

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Chapter 3

MgMfs1, a major facilitator superfamily transporter from the fungal wheat pathogen *Mycosphaerella graminicola*, is a strong protectant against natural toxic compounds and fungicides

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Abstract

MgMfs1, a major facilitator superfamily (MFS) gene from the wheat pathogenic fungus *Mycosphaerella graminicola,* was identified in expressed sequence tag (EST) libraries. The encoded protein has high homology to members of the drug:H^+ antiporter efflux family of MFS transporters with 14 predicted transmembrane spanners (DHA14), implicated in mycotoxin secretion and multidrug resistance. Heterologous expression of *MgMfs1* in a hypersensitive *Saccharomyces cerevisiae* strain resulted in a strong decrease in sensitivity of this organism to a broad range of unrelated synthetic and natural toxic compounds. The sensitivity of *MgMfs1* disruption mutants of *M. graminicola* to most of these compounds was similar when compared to the wild-type but the sensitivity to strobilurin fungicides and the mycotoxin cercosporin was increased. Virulence of the disruption mutants on wheat seedlings was not affected. The results indicate that MgMfs1 is a true multidrug transporter that can function as a determinant of pathogen sensitivity and resistance to fungal toxins and fungicides.

Keywords: MFS transporter; *Mycosphaerella graminicola*; Multidrug resistance; Fungicide; Strobilurin; Cercosporin; Septoria tritici leaf blotch; *Saccharomyces cerevisiae*

Introduction

The ascomycetous fungus *Mycosphaerella graminicola* (Fuckel) Schroeter is the teleomorphic stage of *Septoria tritici* Roberge, the causal agent of septoria tritici leaf blotch of wheat. The fungus is one of the most serious foliar pathogens of wheat in many parts of the world, capable of reducing yields by 30–40% (Polley and Thomas, 1991; Scharen, 1999; Eyal et al., 1987). Disease management relies primarily on breeding for resistance, chemical control, and cultural practices. Despite its economic importance fundamental knowledge on the molecular basis of the plant-pathogen interaction is still limited. The ATP-binding cassette (ABC) transporter MgAtr4 was described as the first virulence factor of this pathogen (Stergiopoulos et al., 2003), and recently also MgSlt2, a mitogen-activated protein (MAP) kinase has been reported as a new pathogenicity factor in *M. graminicola* (Mehrabi et al., 2006).

A transporter classification system (http://www.tcdb.org/), representing a systematic approach to classify transport systems based on their mode of transport, energy coupling mechanism, molecular phylogeny, and substrate specificity distinguishes four major classes of solute transporters; channels, primary (active) transporters, secondary transporters, and group translocators (Busch and Saier, 2002, 2004). Based on this classification, the predicted transport proteins from 141 species, representing the three domains of life, were divided into 134 families, including 7 families of primary transporters, 80 families of secondary transporters, 32 channel protein families, 2 phosphotransferase systems (PTSs), and 13 unclassified families (Ren and Paulsen, 2005).

Nearly half of the solute transporters encoded within the genomes of microorganisms belong to only two families, the ABC and major facilitator superfamily (MFS) of transporters, which are also prevalent in higher organisms (Pao et al., 1998). ABC transporters are primary active transporters, capable of transporting both small molecules and macromolecules upon ATP hydrolysis. Representatives of the pleiotropic drug resistance (PDR) and multidrug resistance (MDR) subfamilies of ABC transporters are well known for their role in resistance to antifungal agents. The second group of major importance, the MFS transporters (Del Sorbo et al., 2000), are secondary carriers

capable of transporting solutes in response to chemiosmotic ion gradients (Paulsen et al., 1996). MFS transporters comprise uni-, sym-, and anti-porters of sugars, peptides, drugs, and organic and inorganic ions. The transporters are typically composed of 400-800 amino acid residues with 12 or 14 transmembrane spanners, and have a molecular mass of 45-90 KDa. Based on sequence homology, functional characteristics, and mechanistic features, the MFS transporters are divided in 17-29 established families as well as five putative families (Pao et al., 1998; Saier et al., 1999). Unlike ABC transporters, MFS transporters have no well-defined characteristic signature. However, a 13-residue amino acid consensus motif present between the second and third transmembrane spanners has been identified and significant sequence homology occurs among all members of the same family (Pao et al., 1998). Based on the full genome sequence of *Saccharomyces cerevisiae*, phylogeny and assigned functions, MFS transporters involved in drug transport are classified in two drug:H^+ antiporter (DHA) families: DHA12 and DHA14 with 12 and 14 predicted spanners, respectively (Sá-Correia and Tenreiro, 2002).

In general, DHA12 and DHA14 MFS transporters can secrete toxic compounds into the outer environment and lower their intracellular concentration. This activity results in a reduced concentration at the target site and in protection against toxic compounds. MFS drug transporters can have low substrate specificity and, therefore, also play a role in sensitivity to drugs such as fungicides. Overexpression of such transporters can lead to MDR of microorganisms (De Waard and Van Nistelrooy, 1979, 1980; Kolaczkowski et al., 1998; Andrade et al., 2000). MDR is defined as the simultaneous resistance of organisms to many unrelated compounds conferred by a single gene, and was first described in mammalian tumor cells resistant to anticancer drugs (Gottesman and Pastan, 1993). In the opportunistic human pathogen *Candida albicans*, the MFS pump CaMDR1 plays a key role in resistance to azole fungicides (Prasad and Kapoor, 2005). Bcmfs1, an MFS multidrug transporter from the plant pathogen *Botrytis cinerea*, is involved in protection against natural toxins and fungicides (Hayashi et al., 2002). For some of the (at least) 23 multidrug MFS transporters identified in the genome of *S. cerevisiae* a role in MDR has been established (Tenreiro et al., 2002, 2005), but the majority are not yet functionally characterized (Sá-Correia and Tenreiro, 2002).

De Waard (1997) postulated that drug transporters of the ABC and MFS superfamilies in plant pathogens can function in secretion of endogenous fungal pathogenicity factors (e.g. toxins) and in protection against exogenous plant defense compounds (e.g. phytoalexins), and therefore, can play a role in virulence. These postulations proved to be valid to both superfamilies of transporters (Stergiopoulos et al., 2002). With respect to MFS transporters the best examples of transporters involved in virulence are TOXA produced by *Cochliobolus carbonum,* and CFP by *Cercospora kikuchii*. TOXA is probably involved in self-protection against HC-toxin and is essential for virulence on maize (Pitkin et al., 1996). CFP mediates secretion of the phytotoxic polyketide cercosporin and contributes to virulence on soybean (Callahan et al., 1999). *CFP* disruption mutants do not produce cercosporin, display a reduced virulence on soybean, and are more sensitive to this compound. For neither of these two transporters, a capacity to transport fungicides has been reported. Replacement of the MFS transporter gene *BcMfs1* in *B. cinerea* did not affect virulence on detached tomato leaves but resulted in increased sensitivity to the natural toxic compound camptothecin, an alkaloid produced by the plant *Camptotheca acuminata*. Hence, it was suggested that BcMfs1 protects *B. cinerea* in host plants with high alkaloid content (Hayashi et al., 2002).

In this paper we describe the functional analysis of *MgMfs1,* the first MFS transporter gene cloned from *M. graminicola* with high homology to MFS transporters with the DHA14 configuration implicated in toxin export or MDR. The results show that MgMfs1 is not likely to be involved in virulence on wheat but acts as a strong multidrug transporter, especially of strobilurin fungicides and fungal toxins.

Materials and methods

Fungal material and culture

Mycosphaerella graminicola IPO323 was used as standard isolate (Kema and Van Silfhout, 1997). Yeast-like cells of *M. graminicola* were either grown in liquid yeast-sucrose medium (YSM: yeast extract 10 g L^{-1}, sucrose 10 g L^{-1}), or liquid yeast-glucose medium (YGM: yeast extract 10 g L^{-1}, glucose 30 g L^{-1}) in a rotary shaker at 140 rpm

and 18 ºC, and on V8 agar plates [50% V8 juice (a commercial beverage consisting of eight vegetable juices), 50% water, 2.5% agar], or potato dextrose agar (PDA: 39 g L^{-1}, Oxoid) at 18 ºC. Mycelium was obtained by diluting a three-day-old culture of *M. graminicola* yeast-like cells grown in YGM to an optical density of 0.05 at 600 nm in liquid Czapek Dox-mycological peptone (CzDMP: 33.4 g L^{-1} Czapek Dox, 5 g L^{-1} mycological peptone) and incubation in a rotary shaker at 140 rpm at 25 °C for three additional days.

Molecular biological techniques

Basic DNA and RNA manipulations were performed based on standard procedures (Sambrook et al., 1989). *Escherichia coli* strain Top10 (Invitrogen) was used for propagation of constructs.

Expressed sequence tags (ESTs) from ten EST-libraries of *M. graminicola* (Kema et al., 2003) were analyzed for the presence of MFS transporters using BlastX homology searches at NCBI.

For library construction, *M. graminicola* genomic DNA was isolated as described by Zwiers and De Waard (2000). The genomic DNA was partially digested with *Bgl*II and size fractionated on 0.4% agarose gels. Fragments ranging in size from 15 to 23 kb were isolated (GFX kit, Amersham Biosciences) and ligated into *Bam*HI-digested λEMBL3 and packaged according to the manufacturer's instructions (Promega EMBL3 *Bam*HI Arms cloning system and packagene system). The genomic library was screened for the presence of the genomic sequence of *MgMfs1* by hybridization with a 1.7 kb *Kpn*I/*Bam*HI fragment derived from the EST clone MgEST7P4K00379 containing *MgMfs1*. DNA from selected phages was cut with *Sal*I and fragments were subcloned in pBluescript for sequencing.

Heterologous expression of *MgMfs1* in yeast

Primers MFS1-E (CCACACCGGTACCACAACC) and MFS1-D (CCTCCTGGAGAATTCCTCCCTCAC) containing artificial *Kpn*I and *Eco*RI restriction sites were used to facilitate the cloning of the full length cDNA sequence of *MgMfs1* in the yeast expression vector pYES2 (Invitrogen). PCR was performed using Platinum *Pfx* DNA polymerase (Invitrogen). Plasmid DNA (100 ng), containing the

MgMfs1 EST fragment was initially denatured for 4 min at 94 °C followed by 35 cycles of 15 s at 94 °C, 30 s at 55 °C, and 2 min at 68 °C. *S. cerevisiae* strain AD12345678 (Δ*yor1*, Δ*snq2*, Δ*pdr5*, Δ*pdr10*, Δ*pdr11*, Δ*ycf1*, Δ*pdr3*, Δ*pdr15*, Δ*ura3*; Decottignies et al., 1998) was transformed with pYES2 containing *MgMfs1* by electroporation. Independent transformants were selected on solid synthetic minimal media containing bacto-yeast nitrogen base without amino acids (6.7 g L^{-1}), drop-out mix (2 g L^{-1} containing amino acids without uracil), galactose (20 g L^{-1}), and noble agar (20 g L^{-1}). The wild-type strain INVSc1 (Invitrogen) transformed with empty vector was used as a control.

Construction of *MgMfs1* disruption plasmid

The primers MFS-G (GTAGTTCTAGACATCCACCG) introducing an *Xba*I site, and MFS-I (TATTTGCTAGCGCCCCATTG) introducing a *Nhe*I site were used to amplify a 1014 bp fragment at the 5' end of the *MgMfs1* EST sequence. A 1096 bp fragment at the 3' end of the sequence was amplified using the primers MFS-J (CTCCTCCTCGAATTCGCCACC) and MFS-H (GTGACAGCAAGCTTCTATCATC), thus introducing an *Eco*RI and a *Hin*dIII restriction site, respectively (Fig. 1). The amplified fragments were cloned in pCR-Blunt (Invitrogen), and subsequently isolated after digestion with the restriction enzymes *Xba*I/*Nhe*I and *Eco*RI/*Hin*dIII, resulting in fragments of 1004 and 1079 bp, respectively. A 71 bp fragment was thus excluded from the middle of the *MgMfs1* open reading frame (ORF).

The pAnH2-5-derived hygromycin resistance cassette cloned in pBluescript II SK (Stratagene) was used as a selectable marker in the plasmid pHygBlue (Van de Rhee et al., 1996; Zwiers and De Waard, 2001). The 1004 bp *Xba*I/*Nhe*I fragment containing the 5' part of the *MgMfs1* ORF was cloned in *Xba*I-digested pHygBlue. Proper orientation of the insert was determined by restriction analysis. The obtained plasmid was digested with *Eco*RI/*Hin*dIII, and ligated to the 1079 bp *Eco*RI/*Hin*dIII fragment containing the 3' part of the ORF. Subsequently, the resulting plasmid was cut with *Xba*I/*Hin*dIII, and the linear construct was cloned in the binary vector pCGN1589 (Zwiers and De Waard, 2001). The obtained vector pCGNΔMfs1 was transformed into *Agrobacterium tumefaciens*.

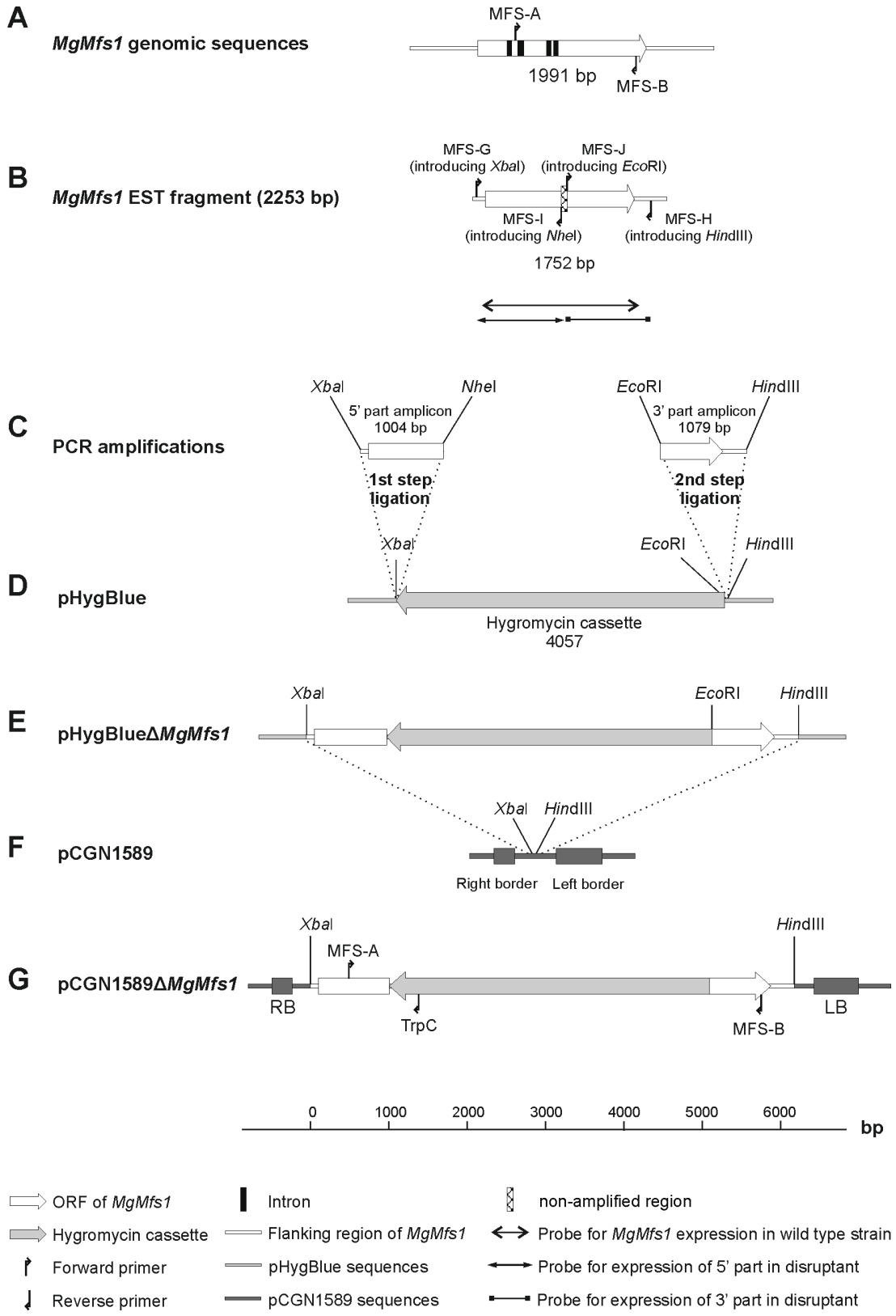
A
MgMfs1 genomic sequences
MFS-A
1991 bp
MFS-B
B
MgMfs1 EST fragment (2253 bp)
MFS-G
(introducing XbaI)
MFS-J
(introducing EcoRI)
MFS-I
(introducing NheI)
MFS-H
(introducing HindIII)
1752 bp
C
PCR amplifications
XbaI
NheI
5' part amplicon
1004 bp
1st step
ligation
EcoRI
HindIII
3' part amplicon
1079 bp
2nd step
ligation
D
pHygBlue
XbaI
EcoRI
HindIII
Hygromycin cassette
4057
E
pHygBlueΔMgMfs1
XbaI
EcoRI
HindIII
F
pCGN1589
XbaI
HindIII
Right border
Left border
G
pCGN1589ΔMgMfs1
XbaI
MFS-A
HindIII
RB
TrpC
MFS-B
LB
0
1000
2000
3000
4000
5000
6000
bp
ORF of MgMfs1
Hygromycin cassette
Forward primer
Reverse primer
Intron
Flanking region of MgMfs1
pHygBlue sequences
pCGN1589 sequences
non-amplified region
Probe for MgMfs1 expression in wild type strain
Probe for expression of 5' part in disruptant
Probe for expression of 3' part in disruptant

Fig. 1. Physical map of *MgMfs1* from *Mycosphaerella graminicola* (A), and schematic representation of PCR strategies used to generate *MgMfs1* disruption construct and to screen disruptants. The primer sets of MFS-G/MFS-I and MFS-J/MFS-H were used to amplify the fragments of 1014 and 1096 bp at the 5' and 3'ends of the *MgMfs1* EST, respectively (B). The subsequent digested fragments of 1004 bp *Xba*I/*Nhe*I and 1079 bp *Eco*RI/*Hin*dIII (C) were inserted into *Xba*I and *Eco*RI/*Hin*dIII sites of the pHygBlue vector (D) during two ligation steps resulting in pHygBlueΔ*MgMfs1* (E). The construct was subsequently excised from the vector using *Xba*I/*Hin*dIII restriction enzymes and inserted into the same sites of the *Agrobacterium tumefaciens* binary vector pCGN1589 (F) generating the disruption construct pCGN1589Δ*MgMfs1*. Multiplex PCR using the forward primer MFS-A and the reverse primers MFS-B and TrpC amplifies a 908 bp band in the true *MgMfs1* disruptants, whereas an additional 1437 bp band appears in the case of ectopic integrations (A, G, and Fig. 3). Northern blots from the wild-type (Fig. 5) were hybridized with a 1776 bp *Kpn*I/*Eco*RI cDNA fragment of *MgMfs1*, and from disruption mutants (Fig. 6) hybridized with cDNA of the full 5' and 3' parts of the gene (B).

Mycosphaerella graminicola transformation and analysis of transformants

Agrobacterium tumefaciens-mediated transformation was performed to disrupt *MgMfs1* in *M. graminicola* strain IPO323 using *A. tumefaciens* strain LBA1100 (Zwiers and De Waard, 2001).

Mitotic stability of hygromycin-resistant colonies was determined by repetitive subculturing on selective and non-selective PDA and YSM. DNA of selected colonies was isolated from freeze-dried material of cells grown in YSM (Raeder and Broda, 1985).

Putative *MgMfs1* disruptants were selected using primers MFS-A (TCCTCCTACCTCCTCAC), MFS-B (CCAACCTCACCCCCATC), and TrpC (AGCAGATCAACGGTCGTCAAGAGA) (Fig. 1). *Taq* DNA polymerase (Invitrogen) was used in PCRs according to manufacturer's instructions and 100 ng genomic DNA was initially denatured for 3 min at 94 °C followed by 30 cycles of 45 s at 94 °C, 30 s at 55 °C, and 90 s at 72 °C.

Sensitivity assays

Minimum inhibitory concentrations (MICs) of compounds were determined for yeast-like growing cells of *M. graminicola* on PDA plates (Zwiers et al., 2003). Q values of compounds were calculated as the ratio between MIC-value of the transformants and the MIC-value of the control strain. Experiments were performed in

duplicate and repeated at least three times. MIC-values were assessed after 10 days of incubation at 18 °C in the dark.

Sensitivity bioassays of *S. cerevisiae* were performed on solid synthetic minimal media amended with toxicants in various concentrations. Cultures of *S. cerevisiae* were grown overnight at 30 °C in liquid synthetic medium and subsequently diluted to an optical density at 600 nm of 0.5 and samples of 5 µl were spotted on plates. Sensitivity to compounds was assessed after incubation at 30 °C for three days.

Compounds tested against *S. cerevisiae* (33) and *M. graminicola* (20), including fungicides, mycotoxins, fungal alkaloids, plant alkaloids, antibiotics, sterols, steroids and dyes are listed in Table 1.

Expression studies and Northern blot analysis

Induction of transcription was studied by adding test compounds to three-day-old yeast-like cell cultures of *M. graminicola* diluted to an optical density of 1 at 600 nm in 30 ml fresh YSM, and to 30 ml three-day-old mycelial cultures. Compounds used were: azoxystrobin, and oxpoconazole (Ube), berberine, camptothecin, cercosporin, cholesterol, corticosterone, cycloheximide, ergosterol, eugenol, neomycin, 4-nitroquinoline-*N*-oxide (4NQO), reserpine, resorcinol, rhodamine 6G, progesterone, and testosterone (Sigma-Aldrich), chlorothalonil, cyproconazole, cyprodinil, and trifloxystrobin (Novartis), 2,4-diacetylphloroglucinol (DAPG; gift of Dr. Jos M. Raaijmakers), imazalil nitrate, and miconazole (Janssen Pharmaceutica), phenol (Invitrogen), and trifluralin (Dow Elanco). Most of the compounds were added to the fungal cultures from a 1000-fold concentrated stock-solution in methanol or DMSO (cercosporin and 4NQO). Hundred-fold concentrated stock-solutions in methanol were used for berberine and cycloheximide, whereas 100-fold stock-solutions in DMSO were used for camptothecin, cholesterol, ergosterol, corticosterone, testosterone and progesterone and a 100 times concentrated aqueous solution of neomycin was used. After addition of compounds, yeast-like cell cultures were incubated at 18 °C and 140 rpm, and mycelial cultures at 25 °C and 140 rpm for 1 h. Cells and mycelium were harvested by centrifugation at 4 °C and 1800*g* for 15 min. The pellets were frozen in liquid nitrogen and stored at -80 °C until RNA isolation.

Table 1. Compounds and concentration ranges used in toxicity assays with *Saccharomyces cerevisiae* and *Mycosphaerella graminicola*

Azole and azole-like fungicides[a]	Strobilurin fungicides	Other fungicides	Antibiotics	Fungal metabolites	Plant metabolites	Dyes/ Steroids/ Sterols
Cyproconazole **0.001-0.1, 0.01-1**	**Azoxystrobin** **0.00001-100, 0.001-1**	Benomyl 0.00005-100	Chloramphenicol 0.001-100	**Cercosporin** **0.000001-10, 0.1-100**	**Berberine** **1-250, 10-500**	Ergosterol 5-150
Epoxiconazole 0.001-0.1	**Kresoxim- methyl** **0.001-100, 0.005-5**	**Bifonazole** **0.001-100, 0.001-10**	**Cycloheximide** **0.01-1, 10-500**	Diacetoxyscirpenol 1-10	**Camptothecin** **1-250, 10-500**	Ethidium bromide 0.001-100
Fenarimol *0.1-10*	**Trifloxystrobin** **0.00001-100, 0.0005-10**	Carbendazim 0.0001-1		Ergocriptine 0.01-1000	Colchicine 0.001-100	Progesterone 5-150
Imazalil *0.001-0.5*		Chloroneb 0.001-100			*8-methoxypsoralen* *0.01-100*	**Rhodamine 6G** **0.1-5, 1-100**
Ketoconazole **0.001-10, 0.01-1**		**Cyprodinil** **0.001-100, 0.01-100**			*Resveratrol* *5-50*	
Miconazole **0.001-0.5, 0.0025-1**		**Fenpiclonil** **0.001-100, 0.1-10**				
Oxpoconazole 0.001-0.1		**Iprodione** **0.001-100, 0.1-10**				
Prochloraz 0.001-1		Naftifine 0.001-100				
Propiconazole 0.001-1		Terbinafine 0.0001-10				
Tebuconazole **0.001-0.1, 0.01-1**		Vinclozolin 0.001-100				

[a] Compounds in regular font were tested only against *S. cerevisiae*, compounds in italic only against *M. graminicola*, and compounds in bold against both organisms. The figures indicate the concentration range of compounds in microgram per milliliter. The first set of concentrations marked in bold is the range used against *S. cerevisiae* and the second set is the range used against *M. graminicola*.

Total RNA of yeast-like cells and/or mycelium of isolate IPO323 and *MgMfs1* disruptants was isolated using the TRIzole® reagent (Life Technologies). RNA (10 µg) was treated with deionized glyoxal, separated on 1.2% agarose gel, and transferred to Hybond-N^+ membranes (Amersham Pharmacia Biotech) by capillary blotting in 10x SSC (Sambrook et al., 1989). Equal loading and transfer of RNA was determined by staining Northern blots with methylene-blue followed by hybridization with a 600 bp *Eco*RI fragment of the 18S ribosomal RNA gene from *Aspergillus niger* (Melchers et al., 1994). Probes used in Northern analysis of the wild-type strain were a 1776 bp *Kpn*I/*Eco*RI, a 1256 bp *Sal*I, and 567 and 689 bp *Sal*I/*Xho*I fragments of *MgMfs1* cDNA. The 1004 bp *Xba*I/*Nhe*I full 5' part of the *MgMfs1* cDNA in disruptants, the 567 bp *Sal*I/*Xho*I fragment from the 5' part, and the 1079 bp *Eco*RI/*Hin*dIII full 3' part were used to study expression of *MgMfs1* in disruption mutants. Probes (25 ng) denatured at 100 °C for 2 min were radioactively labeled using the Prime-a-Gene® Labeling System (Promega) and 3 µl of [α-^{32}P]dCTP (Amersham Biosciences). Northern blots were preincubated in Nasmyth's buffer (18.5% dextran sulphate, 1.85% *N*-lauroyl sarcosine (sarcosyl), 0.011 M EDTA, 0.3 M Na_2HPO_4, 1.1 M NaCl, pH 6.2) at 65 °C for 1 h and, next, hybridized with the probe in the same buffer at 65 °C overnight. Hybridization buffer (10 ml) was made by mixing 5.4 ml solution with 4.6 ml distilled water before use. Blots were washed several times in 2x SSC, 0.1% SDS and 0.1x SSC, 0.1% SDS for 15 min at 65 ºC. The washed blots were exposed at -80 °C for 1-7 days using Kodak® X-Omat AR Film (Sigma-Aldrich).

Virulence assays

Virulence of two independently generated *MgMfs1* disruption strains of *M. graminicola* was compared to the wild-type isolate IPO323 and two independent ectopic transformants (controls) on the susceptible wheat cultivar Obelisk; experiments were repeated four times. Fungal yeast-like cells grown in liquid YSM or YGM media were used at a density of 10^7 cells per ml for inoculation of wheat seedlings as described by Stergiopoulos et al. (2003). Virulence was visually assessed at 21 days after inoculation by evaluation of the leaf area covered with disease symptoms.

Results

Cloning and characterization of *MgMfs1*

BlastX analysis of EST libraries of *M. graminicola* revealed the presence of several MFS transporter sequences with homology to either DHA12 or DHA14 of MFS transporters (Kema et al., 2003). One of these ESTs, named *MgMfs1* (*M. graminicola* major facilitator superfamily transporter 1), was found in five libraries with one clone per library. *MgMfs1* exhibited highest homology to MFS transporters implicated in MDR or toxin transport (Table 2). Two EST clones with *MgMfs1* sequences contained the full length ORF. Southern analysis demonstrated that *MgMfs1* is a single copy gene (data not shown). Screening of a λEMBL3 genomic library of *M. graminicola* with a 1.7 kb *Kpn*I/*Bam*HI EST fragment of *MgMfs1* resulted in the selection of a phage containing a 13 kb insert harboring the full length *MgMfs1* gene. Restriction analysis of the isolated phage DNA resulted in five *Sal*I fragments that were subcloned in pBluescript, and sequenced. Comparison of the genomic DNA sequence with cDNA sequences revealed that *MgMfs1* contains a 1752 bp ORF interrupted by four introns of 58, 70, 55, and 56 bp, respectively. The nucleotide sequence of *MgMfs1* is available at GenBank with Accession No. DQ6619111.

In total 15 kb of genomic DNA was analyzed. This consisted of the isolated phage DNA extended with genomic sequences generated in the *M. graminicola* genome sequence project (available through the trace archive database at NCBI). Approximately 5 kb upstream and 8 kb downstream sequences of *MgMfs1* were analyzed for the presence of neighboring genes. BLAST analysis identified hypothetical genes encoding a putative serin threonin protein kinase upstream of *MgMfs1* and a pyruvate carboxylase downstream of the gene. No synteny was found between the 15 kb *M. graminicola* sequences containing *MgMfs1* and genome sequences of *Fusarium graminearum* and *Magnaporthe grisea*.

Heterologous expression of *MgMfs1* in *Saccharomyces cerevisiae* and bioassays

We transformed the hypersensitive mutant strain AD12345678 of *S. cerevisiae* with full length *MgMfs1* ORF cloned in the yeast expression vector pYES2. The presence

Table 2. Homology of MgMfs1 from *Mycosphaerella graminicola* with MFS transporters from other filamentous fungi

Fungal species	Protein	Function	Accession No.	Identity (%)	Similarity (%)	*E* value
Botrytis cinerea	Bcmfs1	Transporter of natural toxins and fungicides	AAF64435	55	71	1e-171
Aspergillus parasiticus	AFLT	Potential toxin transporter	AAS66020	54	71	2e-143
Aspergillus flavus	AFLT	Aflatoxin efflux pump	AAM53947	54	71	1e-141
Aspergillus fumigatus	-	MFS aflatoxin efflux pump	XP_748188	49	69	8e-138
Aspergillus nominus	AFLT	-	AAS90046	41	63	2e-112
Cercospora kikuchii	CFP	Putative cercosporin transporter	AAC78076	39	58	6e-104
Penicillium citrinum	-	Efflux pump	BAC20568	40	59	6e-99
Cochliobolus carbonum	TOXA	Putative HC-toxin efflux carrier	Q00357	39	60	7e-99

and integrity of the introduced plasmid was checked by restriction analysis. Two independent yeast transformants, ADMgMfs1A and ADMgMfs1B, were used in sensitivity assays. The yeast strain ADYes2 (empty vector control), and strains ADMgAtr1 [expressing the *M. graminicola* ABC transporter *MgAtr1* (Genbank Accession No. AJ243112) (Zwiers and De Waard, 2000; Zwiers et al., 2003)] and INVYes2 (a wild-type strain containing the empty vector) were used as controls. Out of the 33 compounds tested, 22 chemically diverse compounds showed differential activity to both transformants and the control strain ADYes2, indicating a broad substrate range for MgMfs1 (Table 3, Fig. 2). Both independent *MgMfs1* transformants showed exactly the same sensitivity levels for all compounds tested.

MgMfs1 disruption in *M. graminicola*

Disruption of *MgMfs1* was performed using *A. tumefaciens*-mediated transformation. Putative *MgMfs1* disruptants were selected with PCR using a mixture of the forward primer MFS-A, and the reverse primers MFS-B

Table 3. Differential activity of compounds to *Saccharomyces cerevisiae* strains ADYes2 (transformed with the empty vector pYES2), ADMgAtr1 (transformed with *MgAtr1*), and ADMgMfs1A and ADMgMfs1B (independent *MgMfs1* transformants) in agar growth tests

Compounds	Group	MIC-ADYes2[a]	Q-ADMgAtr1[b]	Q-ADMgMfs1[b]
Cyproconazole	Azole	0.01	10	≥ 25
Epoxiconazole	Azole	0.01	2.5	≥ 25
Ketoconazole	Azole	1	1	≥ 25
Miconazole	Azole	0.01	2.5	≥ 100
Oxpoconazole	Azole	0.01	2.5	≥ 25
Prochloraz	Azole	0.1	2.5	≥ 25
Propiconazole	Azole	0.025	4	40
Tebuconazole	Azole	0.01	10	≥ 25
Azoxystrobin	Strobilurin	0.01	1	10
Kresoxim-methyl	Strobilurin	0.01	1	2.5
Trifloxystrobin	Strobilurin	0.01	1	10
Bifonazole	Allylamine	0.25	2	40
Fenpiclonil	Phenylpyrrole	10	1	2.5
Iprodione	Dicarboximide	10	2.5	5
Cycloheximide	Antibiotic	0.05	10	20
Cercosporin	Host-specific toxin	0.01	10	100
Diacetoxyscirpenol	Mycotoxin	1	≥ 25	≥ 25
Berberine	Plant alkaloid	5	2	5
Camptothecin	Plant alkaloid	5	≥ 100	≥ 100
Ergosterol	Sterol	50	2	≥ 5
Progesterone	Steroid	10	2.5	5
Rhodamine 6G	Dye	2.5	1	≥ 4

[a] MIC-value: minimal inhibitory concentration of compounds in µg ml^{-1} for strain ADYes2.

[b] Q value: ratio between MIC-value of compounds for both strains of ADMgMfs1 (A and B) or ADMgAtr1 and ADYes2.

and TrpC. In disruptants the combination of these primers only amplifies a band of 908 bp, whereas in ectopic transformants two bands are amplified, the disruption construct band of 908 bp and the wild-type *MgMfs1* band of 1437 bp (Fig. 1). The PCR screen revealed the presence of six disruptants out of 35 transformants analyzed, representing a single disruption event in 17% of the putative mutants tested (Fig. 3).

Southern blot analysis confirmed the PCR results and indicated that all *MgMfs1*disruptants contained a single copy of the transforming DNA (data not shown). Two independent disruptants, coded IPOΔMFS1A and IPOΔMFS1B, and two ectopic transformants, coded EctMFS1A and EctMFS1B, were selected for phenotypic characterization.

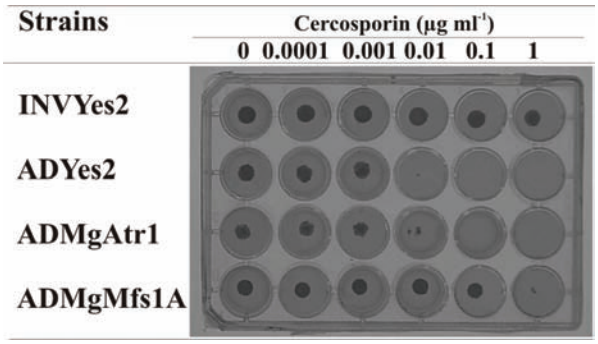

Fig. 2. Effect of heterologous expression of *MgMfs1* from *Mycosphaerella graminicola* in the hypersensitive *Saccharomyces cerevisiae* strain AD12345678 on sensitivity to cercosporin. Strains ADYes2, ADMgAtr1, and ADMgMfs1A transformed with empty vector pYES2, *MgAtr1*, and *MgMfs1*, respectively. The wild-type strain INVSc1 transformed with pYES2 (INVYes2) was used as a control.

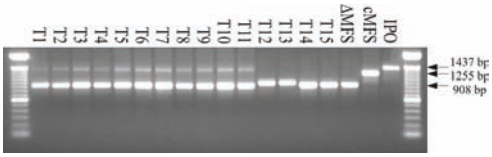

Fig. 3. PCR screen of putative *Mycosphaerella graminicola* mutants (lanes T1-T15) transformed with an *MgMfs1* disruption construct. Forward primer used: MFS-A; reverse primers used: MFS-B and TrpC (Fig. 1A and G). Lane IPO: recipient wild-type isolate IPO323; lane cMFS: *MgMfs1* cDNA fragment; and lane ΔMFS: *MgMfs1* disruption construct. Arrows indicate the 908, 1255 and 1437 bp bands amplified from Δ*MgMfs1*, cDNA of *MgMfs1* and genomic *MgMfs1*, respectively.

Toxicity bioassays with disruption mutants

Based on the results of the yeast sensitivity assays (Table 1), 20 compounds were selected to test their activity against *M. graminicola* IPOΔMFS1A and IPOΔMFS1B. Sensitivity of these *MgMfs1* disruptants to strobilurin fungicides (Fig. 4) and to the host-specific mycotoxin cercosporin (Table 4) was higher than the controls (IPO323, EctMFS1A, EctMFS1B, and the hygromycin-resistant control transformant Sp2). The results indicate that MgMfs1 can protect *M. graminicola* against the toxic activity of these compounds. *MgMfs1* disruptants also exhibited a decreased sensitivity to rhodamine 6G. For none of the other substrates identified in the *S. cerevisiae* assay a phenotype was found in *M. graminicola*.

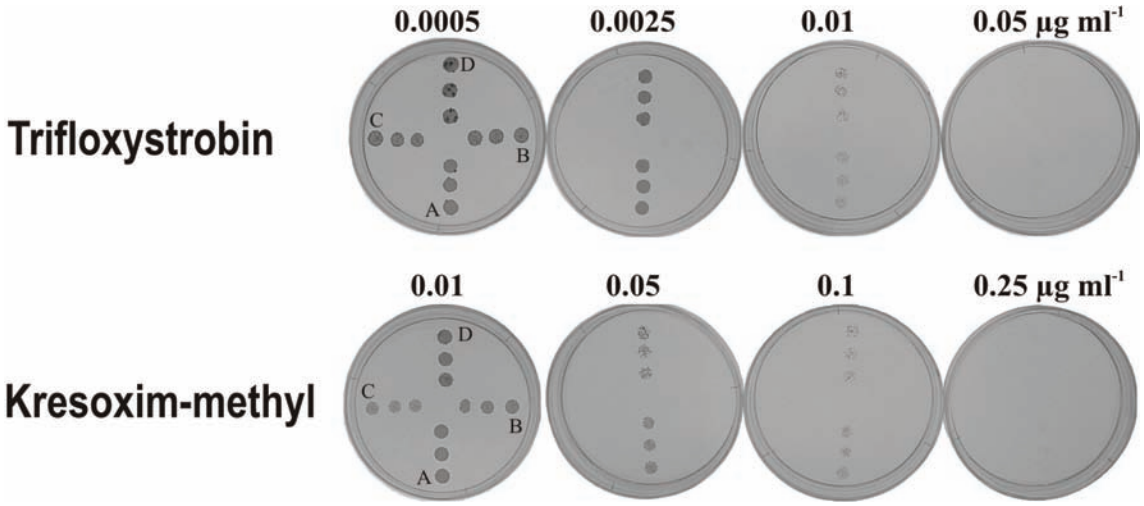

Fig. 4. Sensitivity of *MgMfs1* disruption mutants of *Mycosphaerella graminicola* to the strobilurin fungicides trifloxystrobin and kresoxim-methyl. A, B, C and D indicate the recipient wild-type isolate IPO323, IPOΔMFS1A, IPOΔMFS1B, and EctMFS1A, respectively. Plates were inoculated with drops containing 2000 cells in triplicate, on PDA amended with trifloxystrobin or kresoxim-methyl at concentrations indicated.

Table 4. Differential activity of compounds to *Mycosphaerella graminicola* strains IPO323 (wild-type), EctMFS1A and EctMFS1B (independent transformants of IPO323 with ectopic integration of *MgMfs1*), and IPOΔMFS1A and IPOΔMFS1B (independent disruptants of *MgMfs1*) in agar growth tests

Compounds	MIC-IPO323[a]	MIC-EctMFS1[a]	MIC-IPOΔMFS1[a]	Q-IPOΔMFS1[b]
Azoxystrobin	1	1	0.5	0.5
Kresoxim-methyl	0.25	0.25	0.05	0.2
Trifloxystrobin	0.05	0.05	0.005	0.1
Rhodamine 6G	10	10	25	2.5
Cercosporin	≥ 100	≥ 100	10	≤ 0.1

[a] MIC-value: minimal inhibitory concentration of compounds in microgram per milliliter for strains IPO323, EctMFS1A and B, and IPOΔMFS1A and B.

[b] Q value: ratio between MIC-value of compounds for strains IPOΔMFS1A and B, and strain IPO323 or EctMFS1A and B. Value < 1 indicates increased sensitivity, value > 1 indicates decreased sensitivity.

Expression of *MgMfs1* after treatment with different compounds

The expression of *MgMfs1* in yeast-like cells and mycelium of *M. graminicola* IPO323 was analyzed 1 h after treatment with various compounds from different chemical groups. Out of the 26 compounds tested, the basal level of *MgMfs1* expression was upregulated by 15 compounds. Special attention was given to some compounds for which a phenotype was found in toxicity assays of *S. cerevisiae* or *M. graminicola* disruptants (Fig. 5). The data clearly show that trifloxystrobin, cercosporin and rhodamine 6G, also identified as substrates for MgMfs1 in *M. graminicola*, strongly induced gene expression. Cercosporin and rhodamine 6G induced expression in both morphotypes tested, whereas trifloxystrobin specifically induced expression in yeast-like cells of *M. graminicola*. The results indicate a strong correlation between the potential of MgMfs1 to transport a compound and the ability of that compound to induce the *MgMfs1* expression. Moreover, the results show that the gene is differentially expressed in yeast-like cells and mycelium.

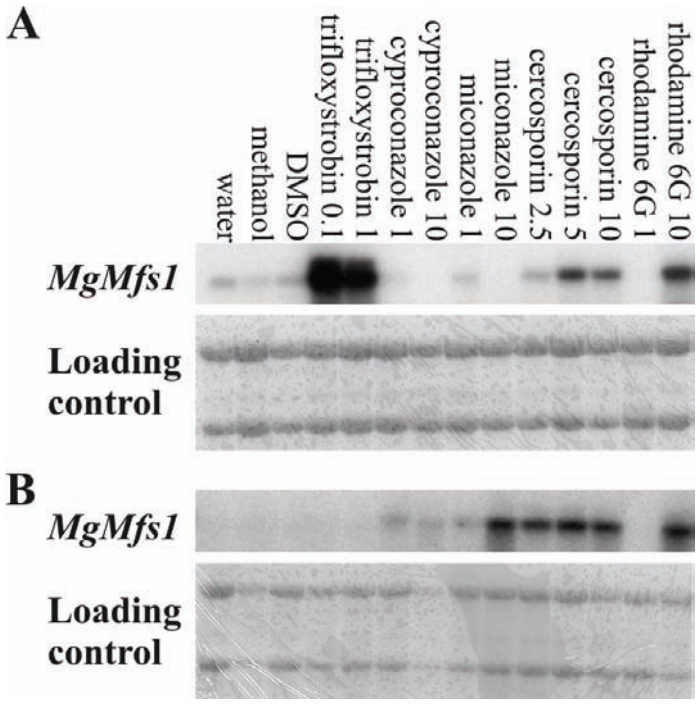

Fig. 5. *MgMfs1* expression in yeast-like cells (A) and mycelium (B) of *Mycosphaerella graminicola* after treatment for one hour with various compounds. Concentrations of compounds are indicated in microgram per milliliter. Water, methanol and dimethyl sulfoxide (DMSO) are used as solvent controls (0.1%). Blots were initially stained with methylene-blue to show the equal loading and transfer of RNA, and then hybridized with a 1776 bp *Kpn*I/*Eco*RI cDNA fragment of *MgMfs1* (Fig. 1B).

Expression of *MgMfs1* in disruptants

In order to analyse expression of *MgMfs1* in the *M. graminicola* disruption mutants IPOΔMFS1A and IPOΔMFS1B, RNA was isolated from cells treated with the strobilurin fungicide trifloxystrobin at concentrations known to induce expression of the gene. Northern blot analyses indicated that disruptants do not produce a full length mRNA. Hybridization with probes from either the 5' or the 3' part of *MgMfs1* showed that expression of the gene in the disruptants is restricted to the formation of a truncated mRNA (Fig. 6).

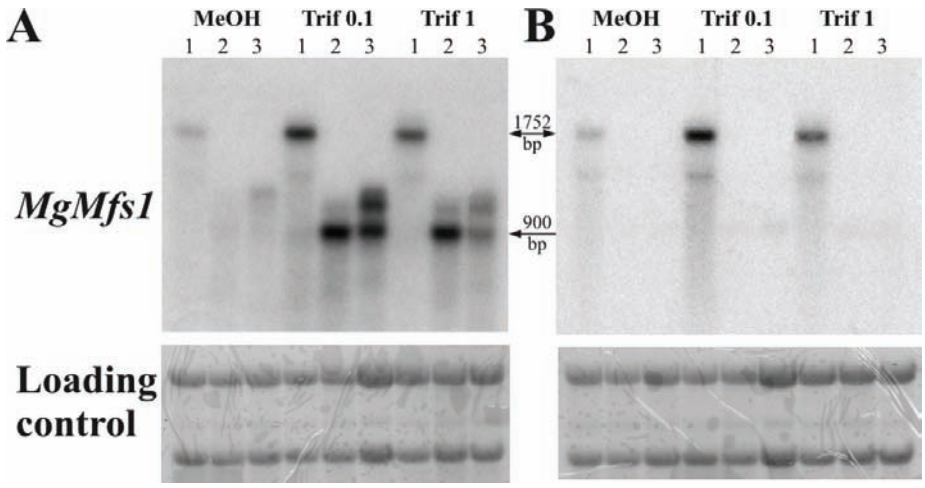

Fig. 6. Effect of disruption on *MgMfs1* expression in *Mycosphaerella graminicola*. Expression of 5' (A) and 3' (B) part of the disrupted gene is shown in IPO323 (1), IPOΔMFS1A (2) and IPOΔMFS1B (3) after 1 h treatment with methanol 0.1% (MeOH), trifloxystrobin 0.1 µg ml^{-1} (Trif 0.1) or trifloxystrobin 1 µg ml^{-1} (Trif 1). cDNA of the full 5' and 3' part of the gene were used as probes (Fig. 1B). Equal loading and transfer of RNA is shown in the stained blots with methylene-blue. Arrows indicate size of full length messenger (1752 bp) and truncated messenger (900 bp).

Virulence assays

Virulence of the *MgMfs1* disruption strains, IPOΔMFS1A and IPOΔMFS1B, was tested on wheat seedlings of the susceptible cultivar Obelisk. No differences in severity and timing of symptom development (necrosis and pycnidia formation) was observed between disruptants and the control strains IPO323, EctMFS1A and EctMFS1B, and Sp2 at 21 days after inoculation (data not shown).

Discussion

This study focused on the identification and functional analysis of *MgMfs1*, the first major facilitator superfamily transporter gene cloned from *M. graminicola*. The gene encodes a DHA14 transporter with high homology to the MFS transporters BcMfs1 from *B. cinerea*, CFP from *C. kikuchii*, and TOXA from *C. carbonum*, which play a role in protection against toxic compounds, particularly fungal toxins (Hayashi et al., 2002; Callahan et al., 1999; Pitkin et al., 1996).

CFP secretes the phytotoxic polyketide cercosporin in *C. kikuchii* and functions in self-protection of the fungus to this toxin. Mutants disrupted in *CFP* do not produce cercosporin, display reduced virulence on soybean, and are more sensitive to this compound (Callahan et al., 1999). Moreover, the introduction of *CFP* into a non-cercosporin producing and cercosporin sensitive fungus resulted in increased cercosporin resistance (Upchurch et al., 2002). The ability of MgMfs1 to protect *S. cerevisiae* against cercosporin suggests that the transporter may play a role in virulence of *M. graminicola* by mediating secretion of host-specific toxins. This hypothesis is supported by the observations that *MgMfs1* expression is upregulated by cercosporin and that *MgMfs1* disruption mutants show increased sensitivity to the toxin. However, a role of a host-specific toxin exerted through MgMfs1 in pathogenesis of *M. graminicola* on wheat has not been established as we did not observe a decreased virulence of *MgMfs1* disruptants on wheat seedlings. Microscopical studies of chlorotic lesions formed upon infection of wheat leaves by *M. graminicola*, suggest that pathogenesis can be associated with secretion of a phytotoxic compound (Kema et al., 1996). Indeed, culture filtrates of *M. graminicola* do contain phytotoxic activity but its significance in pathogenesis remains unclear (Perrone et al., 1999). Therefore, it can not be concluded that MgMfs1 is involved in the secretion of a putative *M. graminicola* toxin involved in pathogenesis. It might be that the lack of such a phenotype is caused by the presence of multiple transporters with overlapping substrate specificity. This lack of phenotype is comparable with that of *BcMfs1* from *B. cinerea*, the closest *MgMfs1* homologue. *BcMfs1* is also capable of transporting cercosporin but it did not function as a virulence factor on tomato (Hayashi et al., 2002).

The strains of *S. cerevisiae* AD12345678 transformed with *M. graminicola MgMfs1*, showed a significant decrease in sensitivity to a wide range of unrelated chemicals, indicating that MgMfs1 is a potent multidrug transporter. Our results demonstrated that MgMfs1 compensates for the absence of major ABC transporters such as PDR5 and SNQ2 in this drug-supersensitive *S. cerevisiae* strain. Multidrug resistance of yeast transformants expressing *MgMfs1* was more pronounced than in mutants expressing *MgAtr1*, the most potent MDR transporter gene characterized from *M. graminicola* so far (Zwiers et al., 2003).

The yeast assay does not necessarily reflect a similar function of MgMfs1 in *M. graminicola* since *MgMfs1* disruption mutants exhibited no phenotype for the majority of the antifungals tested. This discrepancy can be ascribed to redundancy of any transporters resulting in compensation of *MgMfs1* transporter deficiency or to differences in fungal membrane environments influencing the activity of the transporter (Kaur and Bachhawat, 1999). The redundancy of transporters is also likely to be the reason why *MgMfs1* disruption mutants of *M. graminicola* displayed a decreased sensitivity to rhodamine 6G, since the lack of MgMfs1 may be overcompensated by upregulation of other transporters with affinity for rhodamine 6G (Zwiers et al., 2003).

However, *MgMfs1* disrupted mutants of *M. graminicola* showed increased sensitivity to strobilurin fungicides. This indicates that either no redundancy for strobilurin transporters exists or that MgMfs1 is the major strobilurin transporter present in *M. graminicola*. Strobilurins constitute a family of broad-spectrum fungicides that are widely used for septoria tritici leaf blotch control in wheat. This suggests that MgMfs1 can be a determinant of baseline sensitivity of *M. graminicola* to strobilurin fungicides. In addition, overexpression of *MgMfs1* may play a role in development of strobilurin resistance in this pathogen, which developed soon after their introduction in agricultural practice (Fraaije et al., 2003).

Strobilurins are derived from the natural antibiotic strobilurin A, produced by the wood-rotting fungus *Strobilurus tenacellus* (Bartlett et al., 2002). Additionally, the yeast assays identified the antibiotic cycloheximide, and the mycotoxin diacetoxyscirpenol (DAS), produced by *F. graminearum*, as substrates of MgMfs1. MgMfs1 also provided protection in yeast to the toxic plant alkaloids camptothecin and berberine, produced by *C. acuminata* and *Berberis vulgaris*, respectively. These yeast

data combined with the observed phenotype of the *MgMfs1* disruptants to cercosporin and the strobilurin fungicides suggest that MgMfs1 is involved in protection of *M. graminicola* against deleterious compounds produced by antagonistic microorganisms and fungitoxic plant defense compounds. Interestingly, replacement mutants of *BcMfs1* in *B. cinerea*, the closest homologue of *MgMfs1* showed similar phenotypes (Hayashi et al., 2002).

We conclude that MgMfs1 is a strong multidrug transporter and its capability to transport fungal toxins, plant metabolites, and fungicides indicates a potential role in fungicide sensitivity and multidrug resistance, particularly to strobilurin fungicides.

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Chapter 4

The drug transporter MgMfs1 affects sensitivity of the fungal wheat pathogen *Mycosphaerella graminicola* to the strobilurin fungicide trifloxystrobin

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Abstract

The major facilitator superfamily (MFS) drug transporter MgMfs1 of the wheat pathogen *Mycosphaerella graminicola* is known as a potent multidrug transporter with high capacity to transport strobilurin fungicides *in vitro*. We now show that MgMfs1 plays a significant role in sensitivity of this economically important wheat pathogen to the strobilurin fungicide trifloxystrobin. Expression of *MgMfs1* is strongly induced by strobilurin fungicides, and disruption mutants of this gene display a reduced efflux activity of the fungicide *in vitro*, and an increased sensitivity to trifloxystrobin in disease control experiments on wheat seedlings. We also investigated whether overexpression of *MgMfs1* may function as a mechanism of resistance to strobilurin fungicides under field conditions using strobilurin-resistant field strains collected in 2004 in the Netherlands. The high level of resistance to strobilurins in the majority of these strains (80%) could be ascribed to the G143A mutation in cytochrome b, the target site of strobilurin fungicides. However, most of these strains also overexpressed *MgMfs1* and showed a higher trifloxystrobin efflux activity than wild-type strains. For these reasons we suggest that overexpression of *MgMfs1* may be required for normal fitness of strobilurin-resistant strains by preventing accumulation of strobilurin fungicides in fungal membranes and thus safeguarding normal membrane functioning.

Keywords: Leaf blotch; *Septoria tritici*; Disease control; Fungicide resistance; Fungicide efflux; MFS transporter; QoI fungicide; Azole; Cyproconazole

Introduction

The ascomycetous fungus *Mycosphaerella graminicola* (Fuckel) J. Schroeter in Cohn (anamorph: *Septoria tritici* Roberge in Desmaz.) is the causal agent of a major disease on wheat called septoria tritici leaf blotch. This fungus is distributed globally across a wide range of geographical niches, and has gradually emerged as one of the most damaging foliar pathogens of wheat in many parts of the world, especially in areas with high rainfall during the growing season. Total losses of wheat yield due to septoria diseases worldwide are estimated over 9 million metric tons, as annual yield losses during outbreaks can range from 30–40% (Eyal et al., 1987; Palmer and Skinner, 2002).

Disease management relies on breeding for resistance, cultural practices, and chemical control. As the fungus produces airborne sexual ascospores with high potential for dispersal over long distances, random mating (sexual) reproduction of this pathogen leads to populations with a high degree of genetic diversity. The high genetic diversity within the pathogen population can negatively affect the resistance of cultivars by changes in virulence of the pathogen, particularly since wheat cultivars and races of *M. graminicola* show a gene-for-gene relationship (Brading et al., 2002; Chen and McDonald, 1996; Kema et al., 1996; Nelson and Marshall, 1990). Cultural practices aimed to reduce the amount of inoculum available for infections and to reduce the exposure time of the crop to the pathogen constitute the main strategies of septoria disease control, particularly in low crop productivity systems (Bannon and Cooke, 1998).

In wheat cultivating countries where high yields and disease incidence justify additional expenses for disease control, fungicides from different classes of compounds have been used for disease control of septoria tritici leaf blotch. Protective fungicides such as the dithiocarbamates maneb, mancozeb, and zineb and the aromatic fungicide chlorothalonil are effective (Eyal and Wahl, 1975; Hims and Cook, 1992), but have to be applied repeatedly during the growing season, which significantly increases the cost of disease management. Hence, after their introduction in the 1970s, systemic benzimidazole fungicides such as benomyl with curative activity and long protective action were extensively used (Sanderson and Gaunt, 1980). The evolution of

benzimidazole-resistant strains reduced or even completely abandoned the use of these fungicides (Fisher and Griffin, 1984) and led to extensive application of sterol demethylation inhibitors (DMIs) from the 1980s onwards, such as the azole fungicides cyproconazole, epoxiconazole, propiconazole, tebuconazole, and triadimefon. Most azole fungicides are systemic and possess both protective and curative disease control activity (Kuck and Scheinpflug, 1986). Azole fungicides have a site-specific mode of action as they inhibit the enzyme sterol 14α-demethylase involved in sterol biosynthesis (Sisler and Ragsdale, 1984). For that reason, azole fungicides have a risk of resistance development. Although azole resistance emerged relatively slow as compared to other classes of fungicides (De Waard, 1994), the phenomenon occurred in many fungal plant pathogens (De Waard, 1994; Schepers, 1985).

In 1978, an antifungal constituent isolated from the basidiomycetous wood-rotting fungus *Strobilurus tenacellus* was described as strobilurin and subsequently the structure of strobilurin A, a natural fungicidal derivative of ß–methoxyacrylic acid was published (Anke et al., 1984; Schramm et al., 1978). This compound led to the discovery of a new class of broad spectrum systemic fungicides, the strobilurins. The first commercial strobilurin fungicides, azoxystrobin and kresoxim-methyl, were introduced on the market in 1996. Within just four years after introduction of the first strobilurin fungicides into practice several other products were registered and the market share of these fungicides expanded to more than 10% of the total global fungicide market, indicating their high impact for disease control in agriculture. Some strobilurin fungicides efficiently control *M. graminicola* upon protective and curative applications (Bartlett et al., 2002; Godwin et al., 1999; Rohel et al., 2002). The mode of action of strobilurins, also known as quinone outside inhibitor (QoI) fungicides or QoIs, is based on inhibition of mitochondrial respiration by binding at the ubiquinol oxidation centre (Qo site) of cytochrome b and blocking electron transfer between this mitochondrial enzyme and cytochrome c_1 in the cytochrome bc_1 protein complex (Complex III) located in the inner mitochondrial membrane. This results in reduction of ATP production, and disruption of the energy cycle within the fungus resulting in fungal growth inhibition and death (Sauter et al., 1999; Wiggins and Jager, 1994). As strobilurin fungicides are single site inhibitors, a single point mutation in the cytochrome b gene can strongly reduce affinity of the encoded enzyme for strobilurins

and cause fungicide resistance (Grasso et al., 2006). This was rapidly observed in practice. Strobilurin resistance was first reported in the wheat powdery mildew pathogen, *Blumeria graminis* f. sp. *tritici* in Germany in 1998 after a limited number of field applications during one or two growing seasons (Felsenstein, 1999). Indeed, resistance was based on a specific point mutation in cytochrome b (G143A) (Sierotzki et al., 2000b). This G143A point mutation has also been identified in strobilurin-resistant strains of *Pseudoperonospora cubensis* on cucumber (Ishii et al., 2001), *Plasmopara viticola* on grape (Heaney et al., 2000), *Venturia inaequalis* on apple (Steinfeld et al., 2001), and *Mycosphaerella fijiensis* on banana (Sierotzki et al., 2000a).

Strobilurin resistance in *M. graminicola* is also ascribed to the G143A mutation of cytochrome b (Fraaije et al., 2005, 2003; Ware et al., 2006). However, the possibility exists that additional mechanisms play a role as well (Gisi et al., 2002), either alone or in combination with the G143A mutation. This is expected as previous studies demonstrated that ATP-binding cassette (ABC) and major facilitator superfamily (MFS) drug transporters play a role in sensitivity to azole and strobilurin fungicides. ABC transporters are significant for azole sensitivity of laboratory strains of *M. graminicola* (Zwiers et al., 2002), and may account for variations in base line sensitivity of field strains of the pathogen to azoles (Stergiopoulos et al., 2003). The role of MFS transporters in fungicide sensitivity, especially to strobilurins, has become apparent following the characterization of the *M. graminicola* MFS transporter MgMfs1 (Roohparvar et al., 2006). In this study we show in more detail that MgMfs1 functions in strobilurin sensitivity of *M. graminicola in vitro* as well as *in planta*. Our results also suggest that increased efflux activity of this drug transporter safeguards normal membrane function and fitness of strobilurin-resistant strains of *M. graminicola* under field conditions where the G143A mutation is the predominant cause of strobilurin resistance.

Materials and methods

Fungal material and isolation of *M. graminicola* field strains

The *M. graminicola* strains used in this study were the standard field strain IPO323

collected from western Brabant, the Netherlands (Kema and Van Silfhout, 1997) and its derivative transformants IPOΔMFS1A, IPOΔMFS1B and EctMFS1A (Roohparvar et al., 2006), the field strains M1, M3, S190 (Stergiopoulos et al., 2003), and IPO04001, IPO04006, IPO04042, IPO04062, IPO04069, IPO04083, IPO04090 and IPO04093. The latter strains were isolated from wheat leaves with septoria tritici leaf blotch symptoms sampled in seven wheat fields treated with strobilurin fungicides from six locations in the Netherlands in 2004 (Table 1). Pycnidiospores were isolated from leaf segments fixed on microscopic slides after overnight incubation in humid chambers. Pycnidiospores from oozing pycnidia were transferred to plates of potato dextrose agar (PDA: 39 g L^{-1}, Oxoid) supplemented with streptomycin sulfate or ampicillin (50 mg L^{-1}), incubated for a week at 18 °C, and subcultured twice. Pure cultures were grown in liquid yeast-glucose medium (YGM: yeast extract 10 g L^{-1}, glucose 30 g L^{-1}) in a rotary shaker at 18 °C and 140 rpm for five days, and subsequently cultivated on PDA for 3-4 days. Yeast-like cells of *M. graminicola* strains were collected from these plates, and stored in sterile Eppendorf tubes at -80 °C.

Cultural conditions

Yeast-like cells of the strains were either grown in liquid yeast-sucrose medium (YSM: yeast extract 10 g L^{-1}, sucrose 10 g L^{-1}), or liquid YGM in a rotary shaker at 18 ºC and 140 rpm, and on PDA at 18 ºC. Mycelium was obtained by diluting a three-day-old culture of yeast-like cells grown in YGM to a density of 3 x 10^4 cells per ml in liquid Czapek Dox-mycological peptone (CzDMP: Czapek Dox 33.4 g L^{-1}, mycological peptone 5 g L^{-1}) and incubation in a rotary shaker at 25 °C and 140 rpm for three additional days.

Molecular biological techniques

Genomic DNA of *M. graminicola* strains was extracted from 10 mg freeze-dried yeast-like cells using the Puregene DNA Isolation Kit (Gentra systems Inc.). Polymerase chain reactions (PCR) were used to determine the frequency of mating-type idiomorphs (*mat1-1* and *mat1-2*) and the presence of a point mutation in the mitochondrial cytochrome b gene of the strains as described previously (Waalwijk et al., 2002; Ware et al., 2006). The primers *MAT1-1* F and *MAT1-1* R were used to amplify a

Table 1. Field strains of *Mycosphaerella graminicola*

Strains	Number of strains	Location	Cultivar	MIC[a] trifloxystrobin	G143A mutation in cytochrome b	Strains used in this study
Strains collected in the Netherlands in July 2004						
IPO04001-IPO04005	5	Biddinghuizen	Drifter	0.05	-	IPO04001
IPO04006-IPO04007	2	Biddinghuizen	Drifter	>5	+	IPO04006
IPO04008-IPO04030, IPO04033-IPO04038, IPO04095	30	Lelystad	Drifter	>5	+	
IPO04041-IPO04042, IPO04044, IPO04049, IPO04094	5	Lelystad	Chatelet	>5	+	IPO04042
IPO04050, IPO04052	2	Lelystad	Chatelet	0.05	-	
IPO04053-IPO04054	2	Woldendorp	Tadaros	>5	+	
IPO04055-IPO04063, IPO04065	10	Nieuwolda	Chatelet	>5	+	IPO04062
IPO04064	1	Nieuwolda	Chatelet	0.05	-	
IPO04066-IPO04073, IPO04075-IPO04078, IPO04080-IPO04081	14	Delfzijl	Chatelet	>5	+	IPO04069
IPO04074	1	Delfzijl	Chatelet	0.05	-	
IPO04082, IPO04086-IPO04092	8	Groningen	Drifter	0.05	-	IPO04090
IPO04083-IPO04085, IPO04093	4	Groningen	Drifter	>5	+	IPO04083 IPO04093
Strains collected before the introduction of strobilurins						
IPO323	1	Netherlands	Arminda	0.1	-	IPO323
S190	1	Germany		0.01	-	S190
M1	1	France		1	-	M1
M3	1	France		1	-	M3

[a] MIC-value: minimal inhibitory concentration of trifloxystrobin in milligram per liter for growth on PDA.

340 bp fragment in *mat1-1* strains and the primers *MAT1-2* F and *MAT1-2* R to amplify a 660 bp fragment in *mat1-2* strains. A mixture of all four primers was used in a multiplex PCR to generate either a 340 or 660 bp fragment in *mat1-1 or mat1-2*, respectively (Waalwijk et al., 2002). The sense primer StrobSNP2fwd with a mismatch of T in place of G at nucleotide 427 of the cytochrome b gene and the antisense primer StrobSNP1rvs were used to amplify a 639 bp fragment in strobilurin-sensitive strains, while the antisense primer StrobSNPrc7fwd with a mismatch of T in place of G at nucleotide 429 and the sense primer StrobSNPrc1rvs were used to amplify a 302 bp fragment in strobilurin-resistant strains (Ware et al., 2006).

Sensitivity bioassays

Sensitivity of *M. graminicola* field strains to cyproconazole, trifloxystrobin (Syngenta, Stein, Switzerland), and cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) was assessed in agar growth bioassays. Toxicity tests were performed by spotting cells (5 µl of a suspension containing of 4 x 10^5 yeast-like cells per ml), harvested from three-day-old liquid medium on PDA, amended with different concentrations of these compounds. Concentration ranges of cyproconazole, trifloxystrobin, and cycloheximide used were 0.01-1.0, 0.0005-5.0, and 10-1000 mg L^{-1}, respectively. Experiments were repeated twice in triplicate, and minimum inhibitory concentrations (MICs) of compounds were determined by visual assessment after ten days of incubation at 18 °C in the dark.

RNA isolation and Northern blot analysis

Three-day-old yeast-like cell cultures were used to study *MgMfs1* expression in *M. graminicola*. Cell suspensions were diluted to an optical density of 1.0 at 600 nm in 30 ml fresh YSM cultures and incubated for 30 min. These cultures were directly used to investigate the basal expression level of *MgMfs1* in twelve *M. graminicola* field strains with different levels of sensitivity to strobilurin fungicides. *MgMfs1* expression was studied in strain IPO323 5, 15, 30, 60, and 180 min after adding trifloxystrobin from 1000-fold concentrated stock solutions in methanol to final concentrations of 0, 0.001, 0.01, 0.1, and 1.0 mg L^{-1}. Yeast-like cells were harvested by centrifugation at 4 °C and 1800 *g* for 15 min, the pellets were frozen in liquid nitrogen, and stored at -80 °C until

RNA isolation. Frozen cells were disintegrated with a FastPrep Homogenizer FP120 (Thermo Electron Corporation) prior to RNA isolation. Total RNA was isolated using TRIzole® reagent (Life Technologies). RNA (10 µg) was treated with deionized glyoxal, separated on 1.2% agarose gel, and transferred to Hybond-N^+ membranes (Amersham Pharmacia Biotech) by capillary blotting in 10x SSC (Sambrook et al., 1989). Equal loading and transfer of RNA was determined by staining Northern blots with methylene-blue. *Eco*RI/*Kpn*I (1776 bp), *Sal*I (1256 bp), and *Sal*I/*Xho*I (567 and 689 bp) fragments of *MgMfs1* were used as probes. Probes (25 ng) denatured at 100 °C for 2 min were radioactively labeled using the Prime-a-Gene® Labeling System (Promega) and 3 µl of [α-^{32}P]dCTP (Amersham Biosciences). Northern blots were preincubated in Nasmyth's buffer (18.5% dextran sulphate, 1.85% N-lauroyl sarcosine (sarcosyl), 0.011 M EDTA, 0.3 M Na_2HPO_4, 1.1 M NaCl, pH 6.2) at 65 °C for one h and subsequently hybridized to the probe in the same buffer overnight at 65 °C. Hybridization buffer (10 ml) was made by mixing 5.4 ml solution with 4.6 ml distilled water before use. Blots were washed several times in 2x SSC, 0.1% SDS and 0.1x SSC, 0.1% SDS each for 15 min at 65 ºC. The washed blots were exposed at -80 °C for 1-7 days to Kodak® X-Omat AR Film (Sigma-Aldrich).

Accumulation of trifloxystrobin and cyproconazole

Three-day-old mycelial cultures grown in 100 ml CzDMP were harvested by filtering through a 0.85 mm pore sieve. The biomass was collected on a 0.055 mm pore sieve. Mycelium was washed with 50 mM potassium phosphate buffer (pH 6.0, 1% glucose), and resuspended in the same buffer at a density of 6 mg wet weight per ml. Dead mycelium was obtained by boiling the suspension for 10 min and adding chloroform to a final concentration of 0.05%. Mycelial suspensions were incubated for 30 min at 25 °C and 140 rpm. Then, [trifluormethylphenyl-UL-^{14}C] trifloxystrobin (kindly provided by Bayer CropScience, Monheim, Germany) was added from 100x concentrated stock solutions in methanol to initial external concentrations of 0.001, 0.01, 0.1 or 1.0 mg L^{-1} (260 Bq ml^{-1}). Mycelium from cultures (5 ml) was collected by vacuum filtration at time points from 3-125 min after addition of [^{14}C] trifloxystrobin, washed five times with 5 ml phosphate buffer (pH 6.0, 1% glucose), and stored in vials containing LUMASAFE™ PLUS (3 ml; Groningen, The Netherlands). Radioactivity in

the samples was measured with a Beckman LS6000TA liquid scintillation spectrometer (Beckman Instruments Inc, Fullerton, USA). Accumulation of [^{14}C] trifloxystrobin was calculated as pmol trifloxystrobin per mg dry weight of biomass. Efflux of [^{14}C] trifloxystrobin was calculated as the difference in accumulation between dead and living mycelium. Similarly, [^{14}C] cyproconazole (kindly provided by Syngenta, Basle, Switzerland) accumulation was studied at an initial external concentration of 30 mg L^{-1}. The effect of trifloxystrobin on cyproconazole was studied by adding the strobilurin fungicide to the mycelial suspension at a range of concentrations 35 min after the addition of cyproconazole.

***In planta* disease control assays**

Disease control activity of trifloxystrobin on wheat seedlings was tested against two independently generated *MgMfs1* disruption mutants of *M. graminicola*, the wild-type strain IPO323, and an ectopic transformant (controls) in three successive experiments. The susceptible wheat cultivar Obelisk was grown under greenhouse condition in pots containing 15 seedlings (Kema et al., 1996). Eight-day-old seedlings were preventively sprayed with various concentrations of trifloxystrobin (0, 0.5, 1, 2.5, 5, and 10 mg L^{-1}) in 0.01% Tween 20®, allowed to dry for three h, and placed in sealed Perspex-lidded containers overnight. Fungal yeast-like cells grown in liquid YSM or YGM media were harvested by centrifugation at 3,000 *g* for 10 min at 10°C. Pellets were washed once in sterile MilliQ water and resuspended in 0.15% Tween 20® at a density of 10^7 cells per ml. The trifloxystrobin pretreated seedlings were inoculated with these cell suspensions till run off. Inoculated plants were incubated in sealed containers and kept at 20°C in the dark for 48 h and afterwards subjected to a 16 h light photoperiod. The relative humidity in the containers was kept above 80% by placing a water soaked cloth on the bottom of the boxes. Every five days emerging second leaves were clipped to facilitate light penetration and disease assessment. During the foliar spray experiments, seedlings with a different trifloxystrobin treatment were kept at least five m apart in a ventilated corridor in order to restrict the effects of the vapor phase activity of the fungicide used. Disease control was assessed visually by evaluation of the leaf area covered with symptoms 21 days post inoculation (dpi).

Results

Laboratory mutants

Previous *in vitro* studies demonstrated that the *MgMfs1* disruption mutants IPOΔMFS1A and IPOΔMFS1B possess a higher sensitivity to strobilurin fungicides as compared to the wild-type strain IPO323 (Roohparvar et al., 2006). For that reason, the role of MgMfs1 in strobilurin sensitivity of laboratory and field strains of the pathogen was investigated further in expression analysis, accumulation, and *in planta* experiments.

MgMfs1 expression after treatment with trifloxystrobin

Expression of *MgMfs1* in time after treatment with trifloxystrobin at concentrations of 0, 0.001, 0.01, 0.1, and 1.0 mg L^{-1} was analyzed in *M. graminicola* wild-type strain IPO323. Northern blot analysis showed that 0.01 and 0.1 mg L^{-1} trifloxystrobin significantly induced *MgMfs1* transcription (Fig. 1). *MgMfs1* expression levels already increased after 5 min of treatment, indicating rapid induction of the gene. Transcript levels were highest 60-180 min after treatment. Expression levels at concentrations below 0.01 and above 0.1 mg L^{-1} were hardly detectable. The absence of transcripts upon treatment with trifloxystrobin at 1 mg L^{-1} is probably due to a lethal effect of the fungicide, resulting in instant cell death.

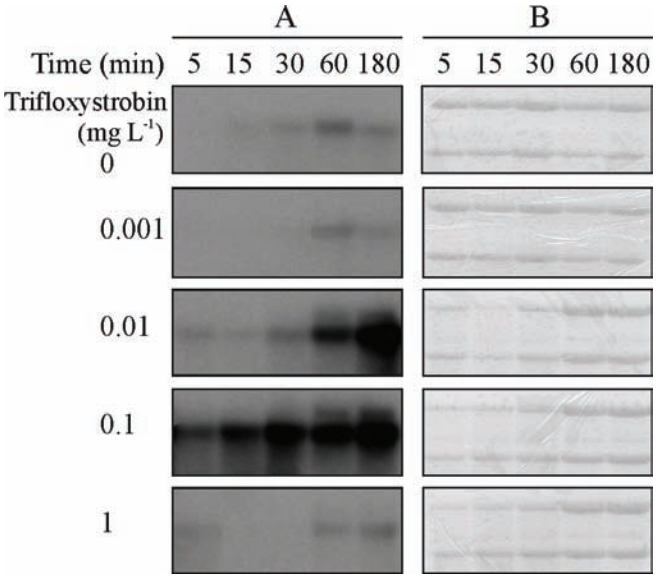

Fig. 1. Expression of *MgMfs1* in time in yeast-like cells of *Mycosphaerella graminicola* strain IPO323 after treatment with trifloxystrobin. (A) Expression after treatment with trifloxystrobin at 0 (methanol 0.1%), 0.001, 0.01, 0.1, and 1.0 mg L^{-1} for 5, 15, 30, 60, and 180 min. The 1256 bp *Sal*I cDNA fragment of *MgMfs1* was used as hybridization probe. (B) Blots stained with methylene-blue showing equal loading and transfer of RNA.

Accumulation of trifloxystrobin in *MgMfs1* disruption mutants

The increased sensitivity of the *MgMfs1* disruption mutants to strobilurins could be due to reduced efflux of these compounds and increased intracellular accumulation of fungicide in mycelial cells. In order to test this hypothesis, the accumulation of trifloxystrobin in cells was measured in time using different initial external concentrations of the fungicide. Accumulation levels of trifloxystrobin in mycelial cells of the wild-type strain IPO323 were almost constant in time, and amounted approximately 0.85, 8.5 and 85 pmol mg^{-1} dry weight at external concentrations of 0.001, 0.01 and 0.1 mg L^{-1}, respectively (Fig. 2A). The results indicate a direct relation between the trifloxystrobin accumulation level in mycelial cells and the initial external concentration of trifloxystrobin. Accumulation of trifloxystrobin in wild-type stain IPO323 and *MgMfs1* disruption mutants was compared at 0.01 mg L^{-1}, since this concentration induced *MgMfs1* expression and was sub-lethal. Trifloxystrobin accumulation by living mycelium of *MgMfs1* disruption mutants was higher as compared to that of the wild-type. In dead mycelium the opposite was found. Accumulation in dead cells minus accumulation in living cells was defined as the amount of fungicide secreted by living cells (Fig. 2B). These values are relatively low in *MgMfs1* disruption mutants as compared to the wild-type IPO323, suggesting that *MgMfs1* deficiency results in decreased trifloxystrobin efflux activity.

Effect of trifloxystrobin on cyproconazole accumulation

In order to test interactions between drug efflux mechanisms for azole and strobilurin fungicides, the effect of trifloxystrobin on accumulation of the azole fungicide cyproconazole, known to be mediated by ABC transporters (Zwiers et al., 2002), was investigated. Cyproconazole accumulation (initial external concentration 30 mg L^{-1}) by *M. graminicola* IPO323 showed a slight decrease in time. Trifloxystrobin added at 0.01 g L^{-1} or higher increased the accumulation of cyproconazole within 10 min after addition of the strobilurin fungicide (Fig. 3). No pronounced differences in the effect of trifloxystrobin at concentrations ranging between 0.01 and 10 mg L^{-1} were observed, indicating a steep dose-response relation between trifloxystrobin concentration and effect on cyproconazole accumulation between 0.001 and 0.01 mg L^{-1}.

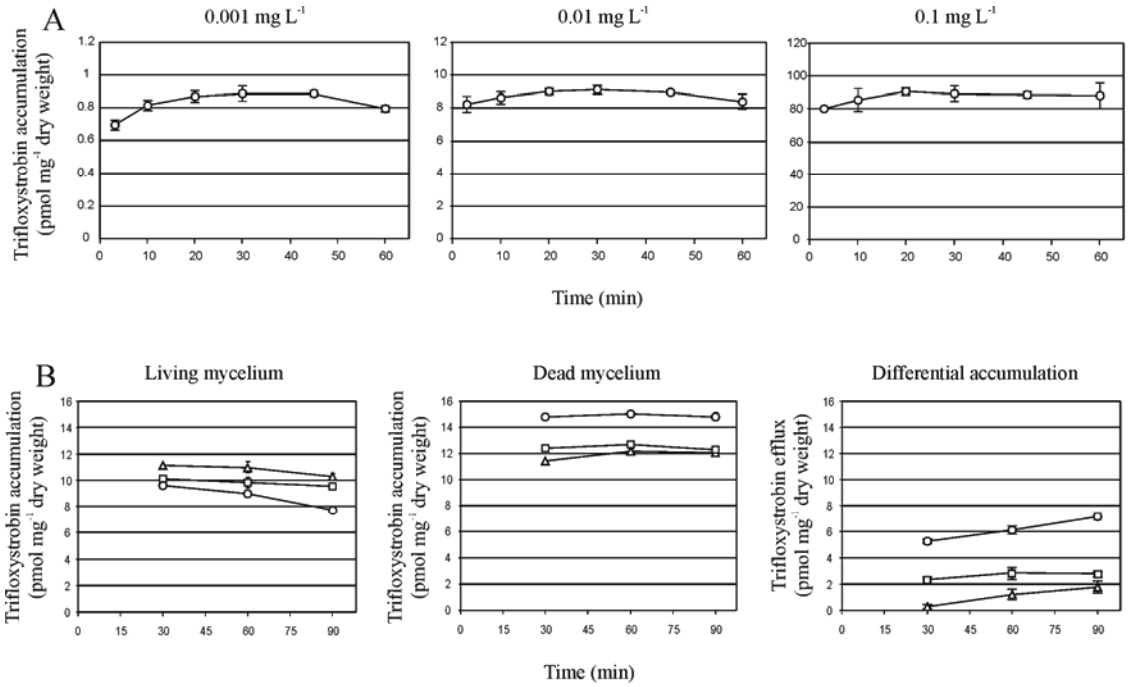

Fig. 2. Accumulation of [^{14}C]trifloxystrobin in time by mycelium of *Mycosphaerella graminicola*. (A) Accumulation by strain IPO323 at initial external concentrations of 0.001, 0.01, and 0.1 mg L^{-1}. (B) Accumulation of trifloxystrobin (0.01 mg L^{-1}) by living and dead mycelium of *M. graminicola*, and efflux of trifloxystrobin in wild-type strain IPO323 (○) and *MgMfs1* disruption mutants IPOΔMFS1A (Δ) and IPOΔMFS1B (□). Bars indicate standard deviation of the means.

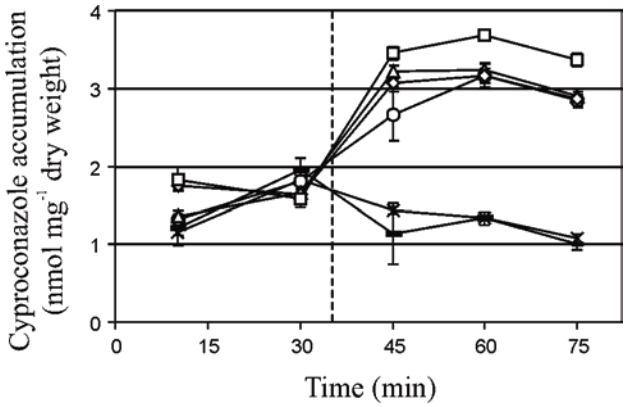

Fig. 3. Effect of trifloxystrobin on accumulation of [^{14}C]cyproconazole (30 mg L^{-1}) by mycelium of *Mycosphaerella graminicola* strain IPO323. Initial trifloxystrobin concentrations 0.001 (x), 0.01 (○), 0.1 (Δ), 1.0 (◊), and 10 (□) mg L^{-1}. Trifloxystrobin was added 35 min after the addition of cyproconazole (dashed line). Methanol 1% (–) was used as control treatment. Bars indicate standard deviation of the means.

In planta trifloxystrobin sensitivity of *MgMfs1* disruption mutants

Efficacy of trifloxystrobin in disease control of septoria leaf blotch incited by the wild-type strain IPO323, *MgMfs1* disruption mutants and the ectopic transformant EctMFS1A was tested on the wheat cultivar Obelisk. All strains showed a similar virulence on untreated seedlings. The visible symptoms emerged at 8-10 dpi as small chlorotic spots near leaf tips and expanded longitudinally across the leaf. Pycnidia developed in the necrotic lesions at 16 dpi. Similar results were observed on seedlings treated with trifloxystrobin at 0.5 mg L^{-1}. However, symptom development on seedlings treated with trifloxystrobin at 1.0 (results not shown) and 2.5 mg L^{-1} by the disruption mutants IPOΔMFS1A and IPOΔMFS1AB was significantly lower than by the control strains (Fig. 4). Trifloxystrobin at 5 and 10 mg L^{-1} fully prevented disease development of all strains tested (results not shown).

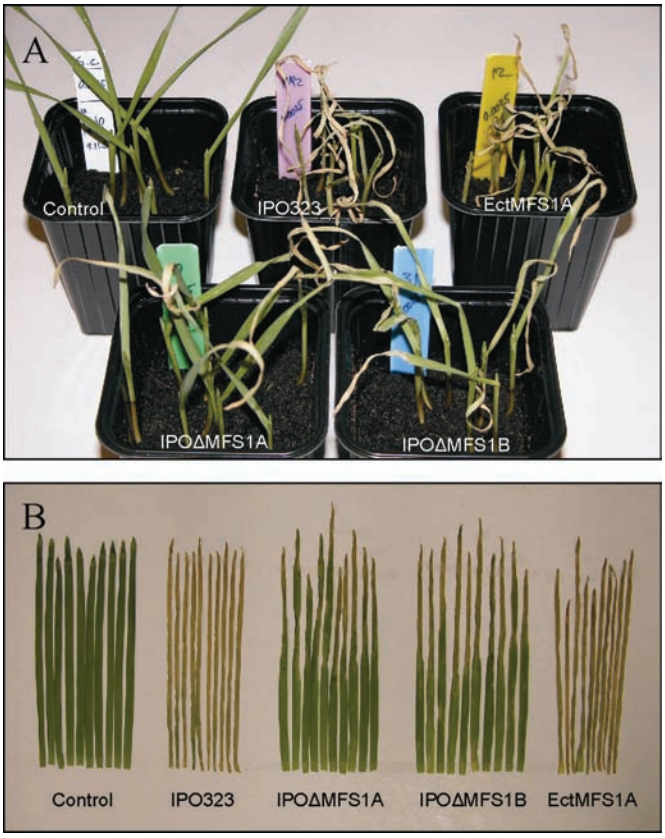

Fig. 4. Differential disease control of various *Mycosphaerella graminicola* strains on wheat seedlings of cultivar Obelisk in preventive foliar spray experiments with trifloxystrobin (2.5 mg L^{-1}). Disease symptoms of the wild-type strain IPO323, *MgMfs1* disruption mutants IPOΔMFS1A, and IPOΔMFS1B and ectopic transformant EctMFS1A are presented on whole plants (A) and detached first emerging leaves (B) at 21 days post inoculation. Control seedlings were sprayed with Tween 20® solutions.

Field strains

In total, 84 *M. graminicola* strains from Dutch wheat fields treated with strobilurin fungicides were collected in 2004. Equal distribution of sampling was tested by determining mating-type idiomorphs of the strains in multiplex PCR assays which amplify 340 and 660 bp fragment of *mat1-1* and *mat1-2* idiomorphs, respectively (Fig. 5A). The assays showed a ratio of 43:41 between *mat1-1* and *mat1-2*, indicating an equal distribution of both mating types.

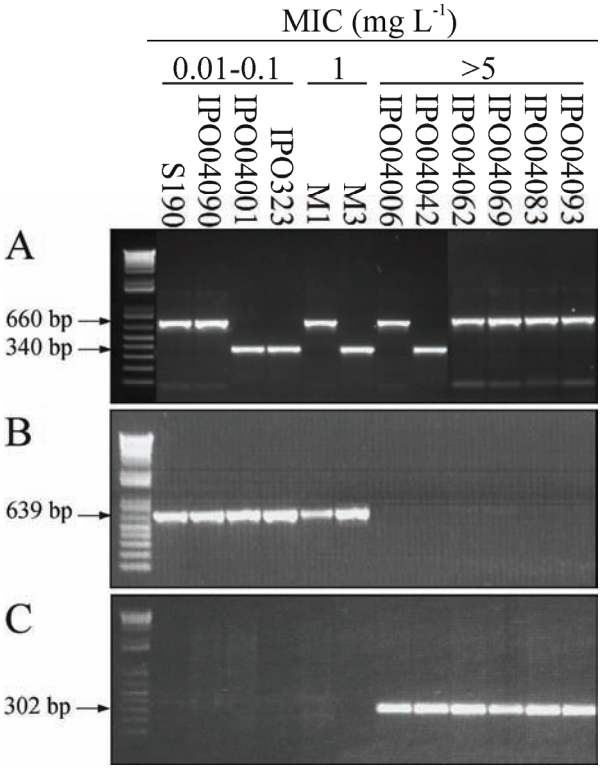

Fig. 5. PCR characterization of *Mycosphaerella graminicola* field strains with different levels of sensitivity to trifloxystrobin for identification of mating-type idiomorphs (A), and absence or presence of G143A mutation in the mitochondrial cytochrome b gene (B and C, respectively). (A) Multiplex PCR results in amplification of a 340 bp fragment in *mat1-1* strains using primers *MAT1-1* F and *MAT1-1* R while primers *MAT1-2* F and *MAT1-2* R amplify a 660 bp fragment in *mat1-2* idiomorphs. (B) Absence of G143A mutation is confirmed by amplification of a 639 bp fragment using sense primer StrobSNP2fwd and antisense primer StrobSNP1rvs. (C) Presence of G143A mutation is confirmed by amplification of a 302 bp fragment using antisense primer StrobSNPrc7fwd and sense primer StrobSNPrc1rvs.

Trifloxystrobin sensitivity

Bioassays showed that 67 out of 84 field strains isolated were highly resistant to the strobilurin fungicide trifloxystrobin, indicating a resistance frequency of about 80%. The mating-type idiomorphs are equally distributed over the resistant strains (results not shown). The MIC-value of trifloxystrobin for the sensitive and resistant strains was 0.05 and >5 mg L^{-1}, respectively, indicating a difference in sensitivity of at least 100-fold (Table 1). Trifloxystrobin sensitivity of field strains collected before the introduction of strobilurins ranged between 0.01 and 1.0 mg L^{-1} (Table 1).

Expression of *MgMfs1*

Basal levels of expression of *MgMfs1* was examined in yeast-like cells of strains with high (IPO323, IPO04001, IPO04090, S190), medium (M1, M3), and low (IPO04006, IPO04042, IPO04062, IPO04069, IPO04083, IPO04093) sensitivity to trifloxystrobin (Fig. 6). *MgMfs1* transcripts were detected in all strains tested. However, strains M1, M3, IPO04006, IPO04062, and IPO04093 displayed relatively high basal expression levels as compared to strains IPO323, IPO04001, IPO04042, IPO04069, IPO04083, IPO04090, and S190, indicating that increased *MgMfs1* expression can be significant for strobilurin resistance.

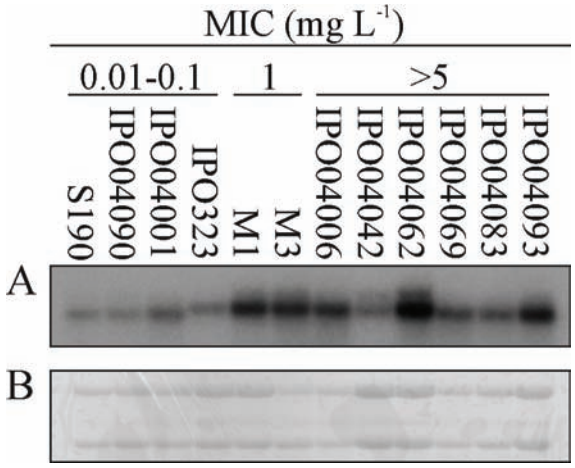

Fig. 6. Basal level of *MgMfs1* expression in yeast-like cells of *Mycosphaerella graminicola* field strains with different levels of sensitivity to trifloxystrobin. (A) Expression is illustrated in sensitive, reduced sensitive, and resistant strains with trifloxystrobin MIC-values of 0.01-0.1, 1, and >5 mg L^{-1}, respectively. The 1256 bp *Sal*I cDNA fragment of *MgMfs1* was used as hybridization probe. (B) Blots were initially stained with methylene-blue to show the equal loading and transfer of RNA.

Trifloxystrobin efflux

Trifloxystrobin efflux in field strains with different trifloxystrobin sensitivity levels was constant in time. Efflux levels in the resistant strains IPO04006 and IPO04042 were significantly higher than in sensitive (IPO323, IPO04090, S190), or reduced sensitive (M3) strains tested (Fig. 7). Mean levels of trifloxystrobin efflux activity in the sensitive (IPO323, IPO04090, S190), reduced sensitive (M3) and resistant (IPO04006, IPO04042) strains were 3-5, 2 and 8-10 pmol trifloxystrobin per mg dry weight, respectively. Other strobilurin-resistant strains tested (IPO04062, IPO04069, IPO04083, IPO04093) also showed an increased efflux activity of trifloxystrobin as compared to strain IPO323 (results not shown).

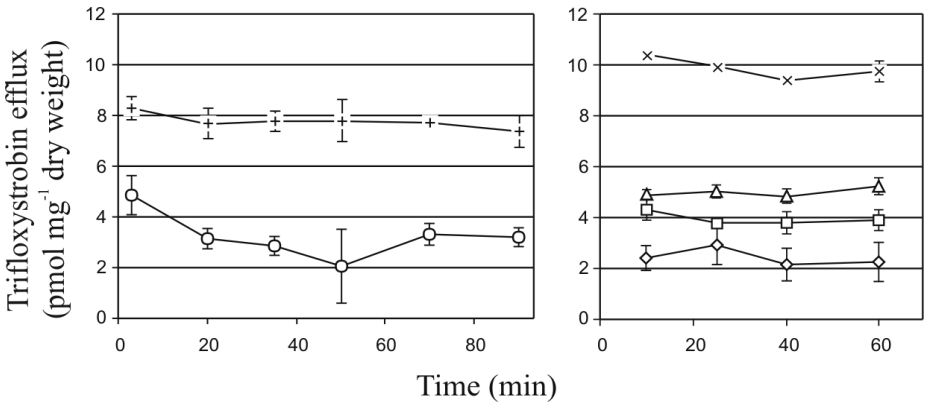

Fig. 7. Efflux of [^{14}C]trifloxystrobin (0.01 mg L^{-1}) by mycelium of *M. graminicola* field strains with different levels of sensitivity to trifloxystrobin. MIC-values of trifloxystrobin for sensitive strains IPO323 (○), IPO04090 (□), and S190 (Δ), reduced sensitive strain M3 (◊), and resistant strains IPO04006 (x), and IPO04042 (+) are 0.01-0.1, 1, and >5, respectively. Bars indicate standard deviation of the means.

Frequency of G143A mutation of cytochrome b

Results of PCR experiments using cytochrome b specific primers demonstrated that a 302 bp fragment, indicative for the single nucleotide mutation G143A, could be amplified with DNA from 67 out of 84 strains tested. The 639 bp fragment of the wild-type cytochrome b gene could be amplified with DNA from the remaining 17 strains (Table 1, Fig. 5B and C). The 67 strains with the G143A mutation and the 17 strains without the G143A mutation are trifloxystrobin resistant and sensitive, respectively (Table 1). These data indicate an absolute correlation between the presence of the G143A mutation and trifloxystrobin resistance, and that about 80% of the collected

strains are strobilurin resistant. PCR assays of the field strains isolated before the introduction of strobilurins all lack the G143A mutation indicating that the observed variation in base line sensitivity to strobilurin fungicides of these field strains is not related to this specific mutation (Fig. 5C).

Resistance to cyproconazole

In order to study simultaneous azole and trifloxystrobin resistance in *M. graminicola*, sensitivity to cyproconazole was tested for 12 field strains randomly selected from strains with wild-type sensitivity, reduced sensitivity and resistance to trifloxystrobin (Table 2). The MIC-value of cyproconazole for the standard reference strain IPO323 with wild-type sensitivity to azole and other fungicides was 0.1 mg L^{-1}. All selected strains with a high degree of resistance to trifloxystrobin had a MIC-value of $\geq$ 5.0 mg L^{-1} indicating that reduced sensitivity to azole fungicides is common in strobilurin-resistant subpopulations of *M. graminicola*. The trifloxystrobin-sensitive strains IPO04001 and IPO04090 collected in 2004 showed a reduced sensitivity to cyproconazole with a MIC-value of 1.0 mg L^{-1}. Other strains isolated from fields with an azole fungicide history, collected before the introduction of strobilurins possessed either a wild-type sensitivity to cyproconazole (M1 and M3) or a slightly reduced sensitivity (S190) with a MIC-value of 0.5 mg L^{-1} for cyproconazole.

Table 2. Cyproconazole sensitivity in *Mycosphaerella graminicola* field strains with differential sensitivity to trifloxystrobin

Trifloxystrobin-sensitive strains[a]		Strains with reduced sensitivity to trifloxystrobin		Trifloxystrobin-resistant strains	
Strain	MIC[b]	Strain	MIC	Strain	MIC
IPO323	0.1	M1	0.1	IPO04006	≥ 5
IPO04001	1	M3	0.1	IPO04042	≥ 5
IPO04090	1			IPO04062	≥ 5
S190	0.5			IPO04069	≥ 5
				IPO04083	≥ 5
				IPO04093	≥ 5

[a] See Table 1.

[b] MIC-value: minimal inhibitory concentration of cyproconazole in milligram per liter for growth on PDA.

Discussion

This study describes that the MFS transporter MgMfs1 can play a significant role in sensitivity of the wheat pathogen *M. graminicola* to the strobilurin fungicide trifloxystrobin. This conclusion is based on the potency of trifloxystrobin to induce expression of *MgMfs1*, the reduced efflux of trifloxystrobin by *MgMfs1* deletion mutants, and the increased sensitivity of these mutants to trifloxystrobin in foliar spray tests. In addition, we found that field strains of *M. graminicola* with resistance to trifloxystrobin may also display enhanced efflux of the fungicide, suggesting that this acquired property is also important for strobilurin-resistant mutants in practice.

The induction of expression of *MgMfs1* in the wild-type strain IPO323 occurred at sub-lethal trifloxystrobin concentrations (0.01-0.1 mg L^{-1}) and after short periods of exposure, indicating that the expression of the transporter indeed responds to the presence of the compound and thus can play a role in fungal protection against this compound. Similar induction patterns have been described for ABC transporter genes from *Aspergillus nidulans*, *Botrytis cinerea*, and *M. graminicola* after treatment with azole and dicarboximide fungicides (Andrade, 2000; Hayashi et al., 2002, 2001; Stergiopoulos et al., 2002; Zwiers and De Waard, 2000). The mechanism of perception of these fungicides by the fungi and the onset of the signaling cascade resulting in increased transcription of these drug transporters is not known. Possible mechanism involved may be binding of the fungicides to substrate binding sites described for transmembrane regions of ABC transporters or an a-specific stress response of the cell membrane due to accumulation of the fungicides in fungal membranes (De Waard et al., 2006). Mitogen-activated protein kinases known as MAPKs involved in transduction of a variety of extracellular signals may operate in signaling cascades that enhance transcription of drug transporter genes (Monge et al., 2006).

Accumulation levels of trifloxystrobin by the *MgMfs1* disruption mutants were higher as compared to the wild-type (Fig. 2B), indicating that the mutants lost fungicide efflux activity. Calculation of the amount of trifloxystrobin secreted by the strains confirmed this assumption (Fig. 2B). We assume that the relatively low trifloxystrobin efflux activity in the mutants can be ascribed to a loss of function of MgMfs1. In a

similar way knock-out mutants of ABC transporter genes in *A. nidulans* and *B. cinerea* displayed increased accumulation and sensitivity levels to azoles (Andrade et al., 2000; Hayashi et al., 2001). Such a clear correlation was not observed for non-functional mutants of ABC transporter genes from *M. graminicola*, possibly because the ABC transporter genes studied only played a minor role in azole sensitivity (Zwiers et al., 2002).

Accumulation studies with the azole fungicide cyproconazole indicated that trifloxystrobin added at sub-lethal and lethal concentrations, enhanced cyproconazole accumulation by *M. graminicola*. Efflux of cyproconazole by the pathogen is probably mediated by multiple ABC transporters (Stergiopoulos et al., 2003) and by the MFS transporter MgMfs1 (Roohparvar et al., 2006). For that reason the effect of trifloxystrobin can probably not be ascribed to competition with cyproconazole for substrate binding sites of all these drug transporter proteins. The most probable explanation for the effect of trifloxystrobin is that inhibition of cytochrome b in mitochondrial respiration results in depletion of energy by which activity of energy-dependent drug transporters is hampered. In this way, the activity of trifloxystrobin is comparable with the effect of the respiration inhibitors sodium azide, CCCP, DCCD, potassium cyanide and oligomycin on accumulation of fenarimol by *A. nidulans* (De Waard and Van Nistelrooy, 1980). We suggest that the increased accumulation of cyproconazole upon trifloxystrobin treatment explains the synergistic interaction of strobilurin and azole fungicide mixtures observed in control of *M. graminicola* and other fungal plant pathogens (Moreau et al., 2005; Stergiopoulos and De Waard, 2002).

Disease control of septoria leaf blotch incited by *MgMfs1* disruption mutants could be achieved at lower dose rates of trifloxystrobin as required for the wild-type strain IPO323. This result indicates that MgMfs1 functions *in planta* under conditions normally used for disease control by trifloxystrobin, and suggests that overexpression of the gene may be a potential mechanism of strobilurin resistance under field conditions. This hypothesis was tested with field strains of *M. graminicola* collected in 2004 from various locations in the Netherlands. The major part of these strains (80%) was highly resistant to trifloxystrobin as demonstrated in *in vitro* bioassays. All resistant strains carried the G143A point mutation as demonstrated by PCR screens. This absolute correlation confirms that the major mechanism of resistance is based on a target site

modification of cytochrome b as described for many other pathogens (Gisi et al., 2002; Grasso et al., 2006) and that the high resistance level of these strains to trifloxystrobin can mainly be ascribed to a single mutation at the target site of the fungicide.

However, trifloxystrobin efflux levels in resistant strains tested were significantly higher than in the sensitive or reduced sensitive strains (Fig. 7). Since our studies with *MgMfs1* disruption mutants identified MgMfs1 as a trifloxystrobin transporter, we hypothesize that enhanced MgMfs1 mediated efflux activity and reduced accumulation at specific target sites can act as an additional resistance mechanism. This hypothesis was strengthened by the observation that at least five out of eight trifloxystrobin-resistant strains tested showed high basal levels of expression of *MgMfs1* as compared to four sensitive strains with a low basal level of expression. We suggest that induced efflux in resistant strains without an increased level of basal expression of *MgMfs1* (strain IPO04042) may be due to overexpression of as yet unidentified drug transporters of strobilurins.

In general, resistance levels in fungi to fungicides mediated by drug transporters are low (Franz et al., 1998; White et al., 2002). Therefore, the contribution of induced efflux in highly strobilurin-resistant isolates to the overall resistance level may be neglectable since it will be masked by the effect of the cytochrome b mutation. However, it may not be excluded that mutations that result in increased efflux mediated by drug transporter genes are required for normal fitness of G143A mutants since the transporter can function as a ‘hydrophobic vacuum cleaner’ of membranes (Gottesman and Pastan, 1993). This potency may be essential for normal fitness of strobilurin-resistant strains since the hydrophobic character of the fungicides may favor membrane accumulation and hamper normal membrane functioning.

Most of the 2004 Dutch field strains tested displayed resistance to both trifloxystrobin and the azole fungicide cyproconazole. The 2004 strains IPO04001 and IPO04090 were resistant to cyproconazole only. These results indicate that resistance to strobilurins and azoles can operate via different mechanisms and should be regarded as multiple resistance. We suppose that the two mechanisms evolved as a result of sequential selection pressure imposed in time by azoles and strobilurins, respectively (Gisi et al., 2000). The absence of cross resistance also corroborates that the main resistance mechanisms operating for strobilurins (changes in affinity for cytochrome b)

and azoles (changes in affinity of sterol C14-demethylase) are different (Gisi et al., 2002; Grasso et al., 2006; Marichal et al., 1999). The degree of cyproconazole resistance in trifloxystrobin-resistant strains collected in 2004 is higher than the degree of cyproconazole resistance in trifloxystrobin-sensitive strains, also collected in 2004 (IPO04001 and IPO04090). This might be explained by assuming that the increased *MgMfs1* expression in trifloxystrobin-resistant strains not only results in increased efflux of trifloxystrobin, but also in increased efflux of cyproconazole and increased reduction of cyproconazole sensitivity. This hypothesis is supported by the observation that heterologous expression of *MgMfs1* in *Saccharomyces cerevisiae* strongly reduces sensitivity to azoles (Roohparvar et al., 2006).

In summary, our results suggest that in laboratory and field strains of *M. graminicola* multiple mechanisms contribute to strobilurin and azole resistance. The major mechanism of resistance to strobilurins in field strains is based on the target site mutation G143A in cytochrome b, as published previously (Fraaije et al., 2005). The frequency of this type of mutants in Dutch field populations is about 80%, suggesting that field control of septoria tritici leaf blotch by strobilurins is severely hampered by strobilurin resistance. An additional resistance mechanism may involve upregulation of *MgMfs1* and possibly other drug transporter genes, resulting in increased efflux of strobilurin fungicides. We suppose that the contribution of this resistance mechanism to the high resistance level of field strains is low because its effect is masked by the high resistance levels due to the cytochrome b point mutation. Still, increased strobilurin efflux activity in these strains may be a condition for normal fitness of highly resistant field strains, since the efflux may prevent strobilurin accumulation in fungal membranes, and hence, safeguard normal membrane functioning.

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Chapter 5

MgAtr7, a new type of ABC transporter from *Mycosphaerella graminicola* involved in iron homeostasis

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Abstract

The ABC transporter-encoding gene *MgAtr7* from the wheat pathogen *Mycosphaerella graminicola* was cloned in a search for additional ABC transporters involved in azole-fungicide sensitivity. Genomic and cDNA sequences indicated that the N-terminus of this ABC transporter contains a motif characteristic for a dityrosine / pyoverdine biosynthesis protein. This makes MgAtr7 the first member of a new class of fungal ABC transporters harboring both a transporter and a biosynthetic moiety. A homologue of *MgAtr7* containing the same biosynthetic moiety was only found in the *Fusarium graminearum* genome and not in any other fungal genome examined so far. The gene structure of both orthologous transporters is highly conserved and the genomic area surrounding the ABC transporter exhibits micro-synteny between *M. graminicola* and *F. graminearum*. Functional analyses revealed that MgAtr7 is neither required for virulence nor involved in fungicide sensitivity but indicated a role in maintenance of iron homeostasis.

Keywords: *Mycosphaerella graminicola*; *Septoria tritici*; ABC transporter; Siderophore; Iron homeostasis

Introduction

All organisms are dependent on transport systems for survival and proliferation in their natural habitat. The uptake and allocation of nutrients by transport systems not only enables metabolism of nutrients but also facilitates regulation of metabolic pathways. Transport systems enable organisms and cells to maintain their homeostatic balance. Furthermore, they are involved in various processes ranging from the establishment and maintenance of electrochemical gradients, inter and intra-species communication, to protection against toxic compounds of endogenous or exogenous origin (Saier, 2000). Depending on the mode of transport and the source of energy used, transporter proteins can be classified in multiple classes and subclasses (Driessen et al., 2000).

One of the best known and characterized families of transporter proteins involved in the protection of organisms against deleterious compounds is the ABC- (ATP binding cassette) transporter superfamily. These transporters belong to the class of primary active transporters using energy derived from the hydrolysis of the diphosphate bond of ATP to drive transport of compounds against a concentration gradient. ABC transporters are present in all organisms, but in eukaryotes their function seems to be limited to efflux of compounds whereas in prokaryotes they are also involved in uptake (Driessen et al., 2000). ABC transporters typically consist of transmembrane (TM) regions and cytoplasmic nucleotide binding fold (NBF) domains. The NBF domains, involved in ATP hydrolysis, contain the Walker A, Walker B motif, and the so-called ABC-signature (Ames et al., 1992; Walker et al., 1982). The ABC signature is highly conserved among ABC transporters and is the feature characteristic for ABC proteins (Croop, 1993). All members of the ABC transporter superfamily have a modular architecture. Based on their composition roughly two types can be distinguished, full-sized and half-sized. Half-sized transporters contain a single NBF fused to a single membrane spanning region whereas in full-sized transporters these domains are duplicated. A further distinction between ABC transporters is based on the position of the NBF domain, either at the N- or C- terminus of the polypeptide, yielding proteins with a $[NBF\text{-}TM]_{1 \text{ or } 2}$ or a $[TM\text{-}NBF]_{1 \text{ or } 2}$ configuration, respectively.

Mycosphaerella graminicola (Fueckel) J. Schrot. in Cohn (anamorph: *Septoria tritici* Roberge in Dezmaz), an ascomycetous pathogen that causes leaf blotch of wheat, is generally recognized as one of the most important foliar wheat pathogens worldwide (Cook et al., 1991; Keon et al., 2005). We are interested in the physiological role of ABC transporters in filamentous fungi in general and in *M. graminicola* in particular. They are implicated in diverse physiological functions such as pathogenesis, fungicide baseline sensitivity, and multidrug resistance (MDR) (De Waard et al., 2006; Stergiopoulos et al., 2002). From a practical point of view the involvement of ABC transporters in baseline sensitivity to sterol demethylation inhibitors (DMIs) and strobilurins, two main classes of fungicides used for control of *M. graminicola*, is of particular interest (Loughman and Thomas, 1992; Roohparvar et al., 2006; Sauter et al., 1999; Zwiers et al., 2002). Several ABC transporters from *M. graminicola* have been characterized with a minor role in protection against azole fungicides (Zwiers et al., 2003, 2002). This contrasts with the situation in *Botrytis cinerea* where BcAtrD was described as the major transporter responsible for the secretion of azole fungicides (Hayashi et al., 2002, 2001).

Several *in vitro* and *in vivo* EST-libraries have been generated yielding over 26000 *M. graminicola* EST sequences (Kema et al., 2003). A search for additional ABC transporters in these EST-libraries led to the discovery of several new *M. graminicola* ABC transporter encoding ESTs. One of these ESTs (*MgAtr7*) exhibited high homology to fungal ABC transporters involved in azole sensitivity and resistance (Hayashi et al., 2002; Kema et al., 2003; Nakaune et al., 1998). The molecular and functional characterization of *MgAtr7* presented in the current paper showed no involvement in fungicide sensitivity but revealed that the gene encodes an extraordinary new hybrid-type of ABC transporter with the $[NBF\text{-}TM]_2$ configuration fused at the N- terminus to a protein domain characteristic for a dityrosine / pyoverdine biosynthesis protein (DIT1_PvcA). Functional analysis showed that this gene has a role in iron homeostasis.

Materials and methods

Fungal material and culture

Mycosphaerella graminicola IPO323 was used as standard isolate (Kema and Van Silfhout, 1997). Yeast-like cells were either grown in liquid yeast-sucrose medium (YSM: yeast extract 10 g L^{-1}, sucrose 10 g L^{-1}), in liquid yeast-glucose medium (YG: yeast extract 10 g L^{-1}, glucose 30 g L^{-1}) in a rotary shaker at 140 rpm and 18 °C or on potato dextrose agar (PDA: 39 g L^{-1},Oxoid) at 18 °C.

Iron-dependent growth was determined in Grimm-Allen (GA) medium as described previously (Baakza et al., 2004). Glassware was soaked overnight in 6M HCl, rinsed with distilled water, autoclaved in the presence of 0.1 % EDTA and again rinsed with distilled water to remove traces of iron. The medium was decontaminated of iron with 8-hydroxyquinoline dissolved in chloroform (1% w/v). The chloroform layer was removed and the medium was washed frequently with chloroform to remove any residual iron-complexes or 8-hydroxyquinoline. Thirty ml of medium in 100 ml Erlenmeyer flasks was inoculated with fungal cell suspensions to an OD600 of 0.05. Ferric iron was added from a 1000-fold stock solution of $FeCl_3$ dissolved in 10 mM HCl. Growth was assessed by measuring the OD600 at 24 hour intervals. Culture filtrates were used for CAS-assays.

Molecular biological techniques

Basic DNA and RNA manipulations were performed based on standard procedures (Sambrook et al., 1989). *Escherichia coli* strain Top10 (Invitrogen) was used for propagation of constructs.

Sequences generated after sequencing of ten EST-libraries of *M. graminicola* (Kema et al., 2003) were analyzed for the presence of ABC transporters using BlastX homology searches.

M. graminicola genomic DNA for library construction was isolated as described by Zwiers and De Waard (2000). Subsequently, genomic DNA was partially digested with *Bgl*II and size fractionated on a 0.4% agarose gel. Fragments ranging in size from 15 – 23 kb were isolated (GFX kit, Amersham Biosciences) and ligated into *Bam*HI-digested

λEMBL3 and packaged according to the manufacturer's instructions (Promega EMBL3 *Bam*HI Arms cloning system and packagene system). The genomic library was screened for the presence of the genomic sequence of *MgAtr7* by hybridization with a 1 kb *Bam*HI / *Pst*I fragment derived from an EST clone containing *MgAtr7* cDNA. DNA from selected phages was cut with *Sal*I, and fragments were subcloned in pBluescript for sequencing.

Total RNA was isolated using the TRIzole® reagent (Life Technologies). RT-PCR was performed on either cDNA made using the universal riboclone® cDNA synthesis system (Promega) or directly on total RNA using the superscript™III one step RT-PCR kit with platinum® Taq (Invitrogen) according to the manufacturer's instructions. Primers used in this study are described in Table 1.

Plasmid constructions

A disruption construct of *MgAtr7* was made using the GPS™-Mutagenesis system (New England Biolabs) and the previously described transprimer donor pGPS3Hyg (Zwiers and De Waard, 2001). The target plasmid pCGNAtr7 was constructed by the insertion of a 4.7 Kb *Xba*I / *Kpn*I cDNA derived fragment containing part of the *MgAtr7* open reading frame (ORF). Screening and analysis of plasmids obtained after the transposition reaction were performed as described before (Zwiers and De Waard, 2001). The selected disruption construct pCGNΔAtr7, with 3.2 and 1.5 kb of flanking homologous DNA, was subsequently transformed into *Agrobacterium tumefaciens* LBA1100 by electroporation and used in *A. tumefaciens*-mediated transformation (ATMT) of *M. graminicola*.

A replacement construct in which the entire DIT1_PvcA moiety and a large part of the ABC transporter moiety was deleted was made in the binary vector pPZPtk8.1 which uses the herpes simplex virus thymidine kinase (*hv-tk*) as negative selection marker (Gardiner and Howlett, 2004). The plasmid pPZPtk8.1-hyg was made by insertion of a 4.1 kb *Eco*RV / *Xba*I fragment containing the hygromycin resistance cassette derived from pGPS3Hyg into pPZPtk8.1 digested with *Hpa*I / *Xba*I. In the next steps the flanking regions were amplified from genomic DNA. A 3.4 kb fragment located upstream of the *DIT1_PvcA-MgAtr7* gene was amplified using primers 7-AA and 7-AF (Table 1). After digestion with *Xba*I this fragment was cloned into pZPtk8.1-

hyg digested with *Xba*I. A 2 kb fragment located at the 3'end of the gene was amplified using primers 7-AD and 7-AE. After digestion with *Hin*dIII the resulting fragment was cloned into the pPZPtk8.1-hyg construct already containing the 3.4 kb insert. Proper orientations were determined by restriction analyses. In the final construct, pZPTk8.1−ΔAtr7, 4.5 kb of *M. graminicola* genomic DNA was replaced by the hygromycin resistance cassette.

Table 1. Primers used in this study

Name	Sequence (5'-3')	Purpose
7-G	TCATTACAAGCTTCTCCGTGGG	RT-PCR analysis
7-R	GCGGTGGTCTGTCGTGGTTTGG	RT-PCR analysis
7-X	GCGAGTGACCCGGATGGAATCT	RT-PCR analysis
7-Y	CTCGCTGGGAAGTGCCACTGAGCTAT	RT-PCR analysis
7-Z	TTGCGGCTTGCGGTCAGTTTCTGGG	RT-PCR analysis
7-AA	CCGAGCCCGTCTAGACATCCGTGGTG	Replacement construct
7-AD	TTCCGAAGACAGACCGGCTACGCTCAG	Replacement construct
7-AE	CGTGCAACGGAAGCTTAGAAAATCGGCT	Replacement construct
7-AF	CTCCGAACTTGTCTAGAGCATACTCCAG	Replacement construct

M. graminicola transformation and selection of transformants

A. tumefaciens-mediated transformation (ATMT) of *M. graminicola* strain IPO323 and the selection of putative disruption mutants after ATMT using pCGNΔAtr7 was performed as described previously (Zwiers and De Waard, 2001). The negative selection method described (Gardiner and Howlett, 2004) was optimized for *M. graminicola* by transferring twelve hygromycin resistant colonies obtained after ATMT with pPZPtk8.1-hyg to PDA with varying concentrations of the thymidine analogues bromo-deoxyuridine (BdU), fluorodeoxyuridine (FdU), and trifluorothymidine (TFT). The sensitivity of the transformants to the nucleoside analogues was compared to the wildtype strain IPO323. Final selection of the hygromycin resistant *MgAtr7* replacement transformants obtained after ATMT with pZPTk8.1−ΔAtr7 was done on PDA medium with FdU (5µM). Transformants were subcultured several times on selective and non-selective PDA to test the mitotic stability. DNA of selected transformants was isolated from freeze-dried material of cells growing in YSM (Raeder and Broda, 1985).

Sensitivity assays

Minimal inhibitory concentrations (MICs) of compounds were determined for yeast-like growing cells of *M. graminicola* on PDA plates (Roohparvar et al., 2006; Zwiers et al., 2003). Strains tested included three independently generated *MgAtr7* disruptants, a hygromycin resistant transformation control strain, and the wild-type recipient isolate IPO323. Compounds used were: azoxystrobin (Ube), berberine, camptothecin, cercosporin, corticosterone, cycloheximide, ergosterol, hydrogen peroxide, resveratrol, rhodamine 6G, and sitosterol (Sigma-Aldrich), bifonazol (Bayer), fenarimol (Dow Elanco), cyproconazole, fenpiclonil and trifloxystrobin (Syngenta), imazalil nitrate, and miconazole (Janssen Pharmaceutica), iprodione (Rhône Poulenc), kresoxim-methyl (BASF) and the bacterial antibiotics 2,4-diacetylphloroglucinol (DAPG), phenazine-1 carboxylic acid (PCA) and phenazine-1-carboximide (PCN) (kindly provided by Dr. J. Raaijmakers, WUR, the Netherlands).

Virulence assays

Virulence of three independently generated *MgAtr7* disruption strains and one *MgAtr7* replacement strain was compared to the wild-type recipient isolate IPO323, and to a hygromycin resistant transformation control strain on the susceptible wheat cultivar Obelisk. Inoculation and virulence assessments were performed as described before (Stergiopoulos et al., 2003b).

Siderophore detection

The detection of siderophores was performed using the universal chrome azurol S assay (CAS) described previously (Schwyn and Neilands, 1987). This assay is based on competition for iron between the ferric complex of the indicator dye and a chelator or siderophore produced by the fungus. Any siderophores produced replace iron from CAS to the siderophore resulting in a measurable colour change of the dye. *M. graminicola* strains were grown for ten days in iron deprived GA-medium. Siderophore production was detected by adding 1 ml of culture filtrate to 1 ml of CAS-assay solution. After color stabilization the absorbance was measured at 630 nm and corrected for the amount of biomass produced. Uninoculated medium was used as negative control. Siderophore detection was also performed after growth in YG-medium under non iron-limiting conditions.

Results

Molecular organization

BlastX analysis of over 26000 ESTs generated from 10 different libraries (Kema et al., 2003) was performed to identify ABC protein-encoding sequences. One of the ABC-transporter-encoding ESTs isolated by this approach, was of particular interest as it showed highest homology to BcAtrD from *B. cinerea* (55% identity) and PMR1 from *Penicillium digitatum* (49% identity). Both BcAtrD and PMR1 are fungal ABC transporters involved in azole-fungicide sensitivity (Hayashi et al., 2002; Nakaune et al., 1998). The tentative gene was named *MgAtr7*.

Screening of a *M. graminicola* genomic library resulted in the selection of a phage harboring a 17 Kb insert. Nine *Sal*I fragments were isolated from this phage and sequenced after subcloning in pBluescript. In the close proximity upstream of *MgAtr7* another putative ORF was identified (Fig. 1). The predicted protein encoded by this ORF contains a motif characteristic for a dityrosine / pyoverdine biosynthesis protein (DIT1_PvcA, Pfam: PF05141). Surprisingly, analysis of the annotated *Fusarium graminearum* genome indicated the presence of an orthologous ABC transporter gene (*FG03735*) also closely associated to a DIT1_PvcA motif. Moreover, the *F. graminearum* genome annotation predicts that *FG03735* encodes a single protein in which the DIT1_PvcA motif is fused to the ABC transporter. Upstream of the putative DIT1_PvcA encoding motif in *M. graminicola* an ORF in a head-to-head orientation was identified on the opposite DNA strand. This ORF exhibits highest similarity to the hypothetical *F. graminearum* gene *FG03736* that is located adjacent to *FG03735* in a similar head-to-head orientation. The *F. graminearum* gene *FG03736* and its *M. graminicola* ortholog contain several motifs characteristic for a non ribosomal peptide synthethase (Fig. 1, Table 2.) The NCBI trace-archive database containing the *M. graminicola* genome sequence was searched using MEGABLAST for sequences enlarging the contig. The original phage derived contig was thus extended to 50887 bps. BlastX analysis of this contig revealed the presence of nine additional ORFs (Fig. 1). Furthermore, this analysis indicated that the synteny between *M. graminicola* and *F. graminearum* is restricted to *FG03735* and *FG03736*. Hypothetical functions of putative genes identified in this contig are listed in Table 2.

Sequence comparison of the genomic *MgAtr7 / DIT1_PvcA* locus with the EST-clones and additionally generated cDNA fragments indicated that the ABC transporter-encoding part of *MgAtr7* contains seventeen introns whereas two additional introns were identified in the DIT1_PvcA encoding part. The annotation of the *F. graminearum* ortholog *FG03735* predicts a nearly similar number of introns, eighteen in the ABC transporter part and two in the DIT1_PvcA part. Moreover, comparison of the intron positions in the *F. graminearum* ortholog *FG03735* with the intron positions of the *M. graminicola MgAtr7* / *DIT1_PvcA* locus showed a striking conservation (Fig. 2). The nucleotide sequence of *MgAtr7* is available at GenBank with accession number EF062310.

Several other fungal genomes were searched for the presence of a DIT1_PvcA motif associated with an ABC transporter. The DIT1_PvcA motif was detected in other fungal genomes, but an association with an ABC transporter was not detected for any of them. Thus the association between an ABC transporter and a DIT1_PvcA motif seems to be rare and restricted to the two wheat pathogens *F. graminearum* and *M. graminicola* (Table 3).

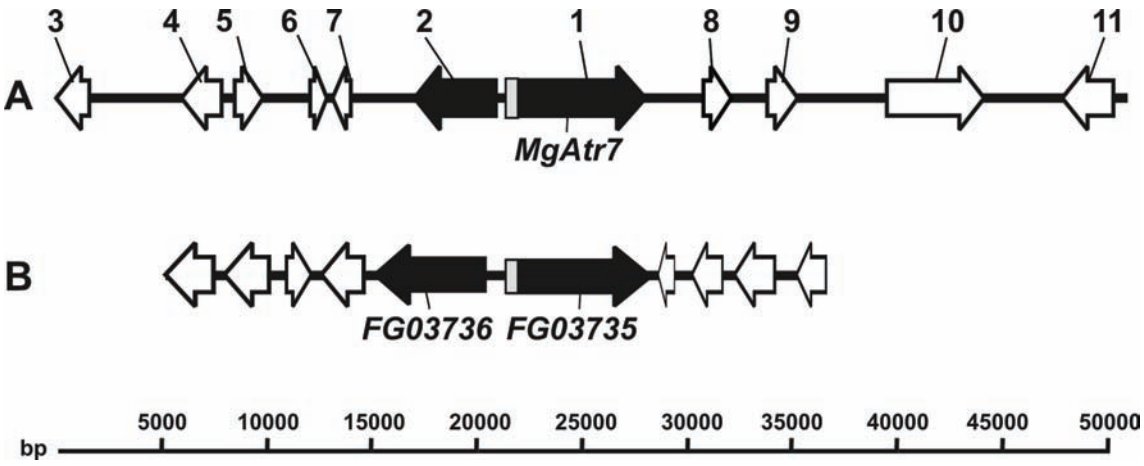

Fig. 1. Comparison of 50887 base pairs of *Mycosphaerella graminicola* IPO323 genomic DNA harboring the *MgAtr7* region (A) with 31554 basepairs of *Fusarium graminearum* PH-1 *FG03735* genomic region (B). Putative ORFs are marked by arrows; the numbers above the *M. graminicola* ORFs are explained in Table 2. Regions with synteny between *M. graminicola* and *F. graminearum* are indicated in black, the predicted DIT1_PvcA motif is marked in grey.

Table 2. BlastP analysis of hypothetical ORFs found in 50887 bps of genomic DNA of *Mycosphaerella graminicola*. Number of ORF refers to number indicated in Fig. 1.

ORF	Closest homologue	*E* value	Organism	Predicted function / motifs
1	FG03735	0.0	*Fusarium graminearum*	DIT1_PvcA motif, ABC transporter
2	FG03736	0.0	*Fusarium graminearum*	NRPS containingAMP-binding domain, phosphoantetheine attachment site and transferase family domain
3	AAA33420	3e-96	*Magnaporthe grisea*	Reverse transcriptase
4	XP_662837	4e-90	*Aspergillus nidulans*	Transposase DNA binding motif, DDE endonuclease motif
5	EAA33012	2e-151	*Neurospora crassa*	Glycolipid 2-alpha-mannosyltranferase like protein
6	EAL91545	2e-85	*Aspergillus fumigatus*	Ubiquinol-cytochrome c reductase iron-sulfur subunit precursor
7	XP_664469	3e-61	*Aspergillus nidulans*	Hypothetical protein
8	BAE61153	4e-67	*Aspergillus oryzae*	Fringe glucosaminyltransferase domain
9	CAD70545	8e-37	*Neurospora crassa*	Glycosyl transferase family domain
10	FG05306	0.0	*Fusarium graminearum*	Serine/threonine kinase catalytic domain, HEAT repeat, WD40 domain
11	CAA67543	6e-76	*Ascobolus immersus*	Reverse transcriptase

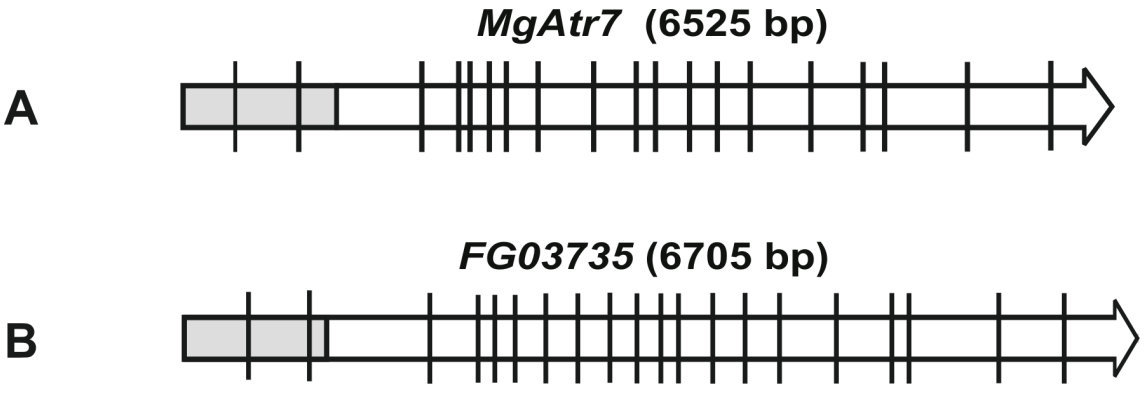

Fig. 2. Comparison of the genomic organization of *MgAtr7* of *Mycosphaerella graminicola* IPO323 (A) with the predicted ORF *FG03735* of *Fusarium graminearum* PH-1 (B). Positions of introns, marked by black vertical lines, are based on cDNA analysis for *M. graminicola* and upon prediction for *F. graminearum*. ORFs are marked by arrows, the DIT1_PvcA motif is indicated in grey.

Table 3. Number of annotated DIT1_PvcA motifs present in fungal annotated genomes. BlastX analyses were used using *Saccharomyces cerevisae Dit1* and the *Mycosphaerella graminicola* DIT1_PvcA moiety of *MgAtr7* to identify DIT_PvcA motifs in fungal genomes. Number identified in *M. graminicola* is based upon Megablast analyses performed on the NCBI trace archive database containing the *M. graminicola* genome sequence.

Fungus	Number of DIT1_PvcA motifs	DIT1_PvcA motifs associated with ABC transporter motifs
Neurospora crassa	0	0
Ustilago maydis	0	0
Botrytis cinerea	1	0
Magnaporthe grisea	1	0
Mycosphaerella graminicola	1	1
Saccharomyces cerevisiae	1	0
Aspergillus nidulans	2	0
Fusarium graminearum	3	1
Stagonospora nodorum	3	0
Aspergillus fumigatus	4	0

MgAtr7 and *DIT1_PvcA* are expressed on the same messenger and part of the same ORF

Based on the prediction that FG03735 would encode a single protein it was decided to study whether the same could be true for *MgAtr7* and the *DIT1-PvcA* motif from *M. graminicola*. RT-PCR with different primer combinations was performed on total RNA isolated from *M. graminicola* grown either as yeast-like cells or mycelium. All results obtained indicated that the DIT1_PvcA encoding motif is present on the same messenger as the ABC transporter encoding part (Fig. 3). This was confirmed by Northern blot analysis showing that a *DIT1-PvcA* specific probe hybridizes to the same fragment as an ABC transporter specific probe (results not shown). Finally, analysis of the sequences derived from cDNA and RT-PCR fragments showed that the predicted *DIT1_PvcA* moiety is part of the same ORF as the *MgAtr7* ABC transporter moiety and thus the two moieties are likely to be translationally fused.

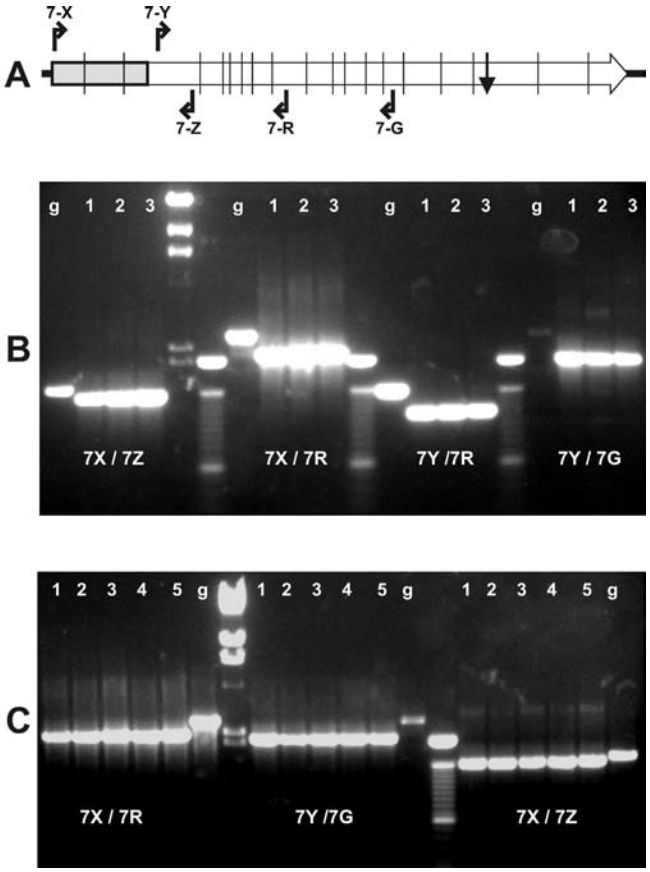

Fig. 3. RT-PCR analysis of *MgAtr7* expression. (A) Scheme indicating the position of the primers used in RT-PCR analyses. Horizontal arrow indicates *MgAtr7*, the DIT1-PvcA motif is indicated as a grey box, the positions of introns are marked by black vertical lines and the position of the insertion of the hygromycin-resistance cassette in *MgAtr7* disruptants is marked by a vertical arrow. (B) Comparison of PCR performed on genomic DNA (g) isolated from wild type *Mycosphaerella graminicola* isolate IPO323 with RT-PCR on RNA isolated from the wild type isolate IPO323 (1) and two independent *MgAtr7* disruptants (2,3) using different primer combinations. (C) RT-PCR analyses using different primer combinations performed on RNA isolated from *M. graminicola* wild-type isolate IPO323 grown in minimal GA-medium without supplemented ferric iron (1) or supplemented with 1,10, 100 µM or 1mM of $FeCl_3$ (2,3,4,5). PCR on genomic DNA (g) of IPO323 was included as control, unlabeled lanes contain DNA marker.

The role of MgAtr7 in fungicide sensitivity and virulence

The sensitivity of *MgAtr7* disruption mutants of *M. graminicola* to azole fungicides and other antifungal compounds was determined. Despite the high homology of MgAtr7 to ABC transporters involved in fungicide sensitivity for none of the tested compounds (see materials and methods) an increase in sensitivity of the *MgAtr7* disruption strains was observed (results not shown).

The virulence of *MgAtr7* disruption and replacement strains was tested on wheat seedlings of the susceptible cultivar Obelisk. No differences in severity and timing of the symptom development between the *MgAtr7* mutants and the wild-type control strain IPO323, and a hygromycin resistant transformation control strain was observed (results not shown).

The role of MgAtr7 in iron homeostasis

The presence of both the DIT1_PvcA and ABC transporter encoding moieties in the same transcript and potentially in the same protein suggests that both components are needed for a similar physiological process. Since the DIT1_PvcA motif is involved in the biosynthesis of the bacterial siderophore pyoverdine the function of MgAtr7 was analyzed in relation to iron homeostasis.

First, it was tested whether MgAtr7 could be involved in the secretion of siderophores produced by *M. graminicola*. The chrome azurol S (CAS) assay (Schwyn and Neilands, 1987) indicated that *M. graminicola* is capable to produce siderophores under iron deplete growth conditions, whereas siderophore production is undetectable in iron containing or iron repleted liquid media. CAS-assays performed on culture filtrates of strains containing a disrupted or deleted *MgAtr7* indicated that siderophore production was unaffected in these mutants (results not shown).

Second, the growth of the wild type isolate IPO323, transformants in which *MgAtr7* was disrupted, and transformants in which 4.5 kb of *MgAtr7* was replaced, was measured during growth in a rich medium and during growth in minimal GA medium containing varying iron concentrations. No growth differences were observed when isolates were grown in rich YG-medium. When grown in minimal GA-medium amended with varying iron amounts growth of the wild type was stimulated in the presence of exogenously supplied ferric iron, with an optimal growth at 100 µM Fe. This contrasts with the *MgAtr7* disruption and replacement mutants. In these mutants no obvious stimulatory effect on growth of low Fe concentrations (1-10 µM) could be observed. Moreover, 100 µM Fe started to limit growth in the *MgAtr7* mutants which was even more apparent at 1mM of ferric iron (Fig. 4).

Finally, RT-PCR data presented in Fig. 3C indicate that *MgAtr7* is expressed during growth in minimal media supplemented with varying concentrations of ferric iron.

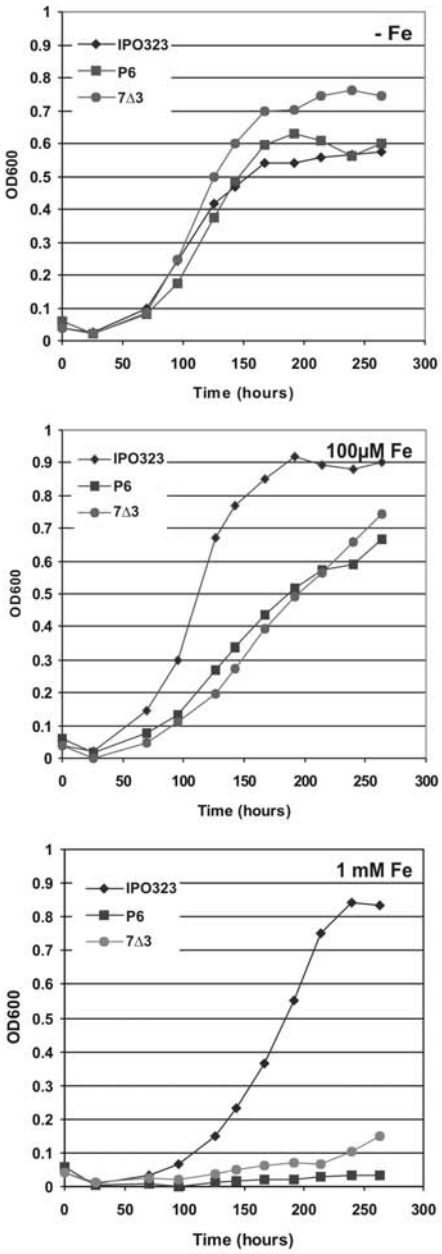

Fig. 4. Growth of *Mycosphaerella graminicola* strains containing a wild-type (IPO323), deleted (P6), or disrupted (7Δ3) *MgAtr7* locus monitored in GA-minimal medium depleted of ferric iron (-Fe) or supplemented with 100 µM or 1 mM $FeCl_3$.

Discussion

ABC transporters play an important role in fungicide sensitivity and resistance (De Waard et al., 2006; Lage, 2003). Analysis of laboratory-generated mutants of the five *M. graminicola* ABC transporters characterized to date, demonstrated that they can function in baseline sensitivity and resistance to azole fungicides (Stergiopoulos et al., 2003a; Zwiers et al., 2002). However, none of the characterized transporters could be classified as the main drug-transporter determining azole-sensitivity in *M. graminicola*.

Therefore, we screened ten *M. graminicola* EST-libraries for the presence of homologues of fungal ABC transporters involved in azole-fungicide sensitivity, resulting in the discovery of *MgAtr7*. However, no function for *MgAtr7* in fungicide sensitivity could be attributed. This contrasts to the situation in *Aspergillus nidulans* where a similar functional search resulted in the identification of the azole transporter AtrG (Andrade, 2000). A similar approach to identify ABC transporters involved in pathogenicity has recently led to the isolation of the ABC transporter gene *FcABC1* from *Fusarium culmorum* (Skov et al., 2004). Our functional homology search in *M. graminicola* was less successful, indicating that orthologs do not necessarily perform similar functions.

The genomic organization surrounding *MgAtr7* was examined in more detail and revealed a DIT1_PvcA encoding motif upstream of the *MgAtr7* ORF. Surprisingly, the closest homolog of *MgAtr7*, the *F. graminearum* locus FG03735 also contains a DIT1_PvcA motif. The conserved gene structure and exhibited micro-synteny between *M. graminicola* and *F. graminearum* were only found in these two wheat pathogens, suggesting a common function or common origin (Fig. 1 and 2**)**. This is reminiscent of an ancient gene cluster that has been deleted / rearranged in other fungi or of a cluster recently and independently evolved in both fungi. However, the striking conservation of both intron position and intron number in both fungi suggests that independent evolution is not likely. It might also be possible that this micro-syntenous region has been introduced by horizontal gene transfer in one or both fungal species. The fact that in *M. graminicola* both downstream and upstream of the micro-syntenous region areas with high homology to reverse transcriptases and fungal transposons can be distinguished (Table 2) might suggest horizontal transfer.

The RT-PCR and cDNA analyses described show that both motifs are expressed on the same messenger and are part of the same ORF. This implies that MgAtr7 has an extraordinary topology consisting of an ABC transporter N-terminally fused to a Dit1_PvcA motif. Thus, MgAtr7 would be the first example of an ABC transporter translationally fused to a putative biosynthetic moiety. An analysis at protein level should give the definite answer whether *MgAtr7* encodes such a hybrid protein or not. Another explanation for the fact that both motifs are expressed on the same messenger might be that they are part of a dicistronic messenger, encoding two independently

translated proteins. Although the majority of eukaryotic genes are monocistronically expressed and under the control of their own unique promoter, in the last two decades accumulating data indicate that the operon model also applies to eukaryotes. One type of eukaryotic operons, involves dicistronic transcription resulting in translation of the dicistronic messengers without any additional processing of the messenger (Andrews et al., 1996; Garcia-Rios et al., 1997; Lee, 1991). In fungi data about dicistronic expression is limited. Until now dicistronic expression has only been reported for small nucleolar RNAs in *Saccharomyces cerevisiae* and for the cercosporin toxin resistance gene (CRG1) from *Cercospora nicotianae* (Chanfreau et al., 1998; Chung et al., 2003).

Whether the DIT1-PvcA motif and the ABC transporter are part of one protein or not, their co-regulated expression suggests that they play a role in the same physiological process. In *S. cerevisiae* DIT1 is a sporulation-specific enzyme catalyzing the reaction from L-tyrosine to dityrosine. Dityrosine is part of bisformyl dityrosine, an ascospore cell wall insoluble macromolecule transported by the major facilitator superfamiliy transporter Drt1p, and essential for resistance of spores to unfavorable environmental conditions (Briza et al., 1994; Felder et al., 2002). The *PvcA* gene encodes a homolog of DIT1 and is implicated in the condensation of L-tyrosine and L-2,-4-diaminobutyric acid (DAB) a proposed step in the biosynthesis of the *Pseudomonas aeruginosa* siderophore, pyoverdine (Stintzi et al., 1999). The *F. graminearum* gene FG03736 and its *M. graminicola* ortholog found within the microsyntenous region both contain protein motifs characteristic for a non ribosomal peptide synthetase (NRPS) (Fig. 1). As the deduced protein of this ORF has also limited homology to MxcG, a myxobacterial NRPS involved in the biosynthesis of myxochelin type iron chelators (Silakowski et al., 2000) taken together these data suggest that MgAtr7 might function in siderophore production and iron homeostasis.

The results of the CAS-assays with *MgAtr7* mutants exclude a direct role of MgAtr7 in siderophore production and or siderophore secretion. However, as *MgAtr7* mutants were affected in their response to exogenously supplied iron, the presence of MgAtr7 seems to modulate the availability of iron in the cell. This can be explained by assuming that the Dit1_PvcA moiety and the putative NRPS are (partly) responsible for the synthesis of a compound which is subsequently secreted by the MgAtr7 transporter moiety to the outside of the cell. In this view MgAtr7 would be analogous to both DIT1

and Drt1p of *S. cerevisiae*. Binding of iron to this putative cell-wall bound compound could not only protect the fungus against toxic levels of iron, but also provide a supply of iron to be used under limited iron supply. This resembles the “feast or famine” situation in mycobacteria where two types of siderophores can be produced: the cell envelope bound mycobactins and the soluble exochelins and carboxymycobactins. The mycobactins are considered to play a role in short-term storage of iron for its subsequent transfer into the cell mediated by the exochelins or other iron transporting mechanisms including ABC transport systems (Kunkle and Schmitt, 2005; Ratledge and Dover, 2000; Rodriguez and Smith, 2006).

In summary, we have identified MgAtr7 as a novel type of ABC transporter which seems to be unique for *M. graminicola* and *F. graminearum*. The transporter is neither involved in virulence nor in protection against fungicides or other xenobiotics. However, our data suggest that MgAtr7 might function in fungal iron homeostasis by modulating iron levels in the cell.

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Chapter 6

Control of *Mycosphaerella graminicola* on wheat seedlings by medical drugs known to modulate the activity of ATP-binding cassette transporters

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(Submitted)

Abstract

Medical drugs known to modulate the activity of human ATP-binding cassette (ABC) transporter proteins (modulators) were tested for their ability to potentiate the activity of the azole fungicide cyproconazole against *in vitro* growth of *Mycosphaerella graminicola* and to control disease development of the pathogen on wheat seedlings. *In vitro* modulation of cyproconazole activity could be demonstrated in paper disc bioassays. Some of the active modulators (amitriptyline, flavanone and phenothiazines) increased the accumulation of cyproconazole in *M. graminicola*, suggesting that they reversed cyproconazole efflux. However, synergism between cyproconazole and modulators against *M. graminicola in planta* could not be shown. Despite their low *in vitro* toxicity to *M. graminicola*, some modulators (amitriptyline, loperamide, and promazine) did show a significant intrinsic disease control activity in preventive and curative foliar sprays. The results suggest that these compounds have an indirect disease control activity based on modulation of fungal ABC transporters essential for virulence and constitute a new class of disease control agents.

Keywords: Amitriptyline; Azoles; Cyproconazole; Disease control agents; Drug efflux; Flavanone; Fungicides; Loperamide; Modulators; Phenothiazines; Synergism

Introduction

Plant pathogenic fungi possess various mechanisms to cope with the activity of natural toxic compounds that may occur in their living environment. These compounds can either be antibiotics produced by other microorganisms or plant defence compounds present in host plants, such as phytoalexins and phytoanticipins. These mechanisms may involve the evolution of insensitive target sites, compartmentalisation, and metabolism of toxic compounds into non-toxic products. Another mechanism operating in many organisms involves reduced accumulation of toxic compounds at their target site due to secretion by ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters (De Waard, 1997; Stergiopoulos et al., 2002). These transporters are membrane pumps that can transport a wide variety of natural products, including plant antimicrobials. Transport mediated by these transport systems into the outer environment can significantly reduce the intracellular concentration of toxicants and protect organisms with a sensitive target site against toxic activity of these compounds. The validity of this mechanism of natural insensitivity to natural toxic products has first been demonstrated in studies with a mutant of *Staphylococcus aureus* that lacks the multidrug pump NorA (Hsieh et al., 1998). The same mechanism has been described for the ABC transporters BcAtrB and GpAbc1 from the plant pathogens *Botrytis cinerea* and *Gibberella pulicaris* which function as virulence factors of these pathogens by providing protection against the plant defence products resveratrol in grapevine and rishitin in potato, respectively (Fleissner et al., 2002; Schoonbeek et al., 2001). The importance of ABC transporters in virulence has also been established for ABC transporter mutants of *Magnaporthe grisea* and *Mycosphaerella graminicola* on rice and wheat, respectively. However, the plant defence compounds acting as substrate for these transporters have not yet been identified (Stergiopoulos et al., 2003b; Urban et al., 1999). MFS transporters can also mediate natural insensitivity to plant defence compounds as shown for Bcmfs1 from *B. cinerea* and MgMfs1 from *M. graminicola* (Hayashi et al., 2002; Roohparvar et al., 2006).

A second physiological function of ABC and MFS transporters from plant pathogens is the secretion of endogenous toxic products that are relevant for saprophytic

survival and virulence on host plants. Known examples are MFS transporters of *Cercospora kikuchii*, *Cochliobolus carbonum*, and *Fusarium sporothichioides* that mediate the secretion of cercosporin, HT-toxin, and trichothecene, respectively (Alexander et al., 1999; Callahan et al., 1999; Pitkin et al., 1996). Activity of these transporters also functions in self-protection against the toxins. ABC transporters with a similar role in virulence of plant pathogens have not been reported, but it is expected that they do exist.

ABC and MFS transporters belong to one of the largest protein families. The redundancy of the transporters and their broad and overlapping substrate specificity explains why these proteins are also able to transport xenobiotics over biological membranes. These xenobiotics include medical drugs, antibiotics, antimycotics and agricultural fungicides (Ambudkar et al., 1999). In this respect, ABC transporters became particularly known for their role in multidrug resistance (MDR), which is the simultaneous development of resistance against multiple unrelated chemicals. This phenomenon has first been demonstrated in cancer cells after prolonged treatment with anticancer drugs. MDR can be based on overexpression of specific ABC transporters that prevent accumulation of the drugs in cancer cells to effective concentrations at their target sites (Gottesman and Pastan, 1988). At present, MDR is also a serious threat to effective control of bacterial and fungal diseases in mammalians and a potential risk in chemical control of agricultural pests and diseases (De Waard et al., 2006).

The important role of ABC transporters in MDR of cancer cells has led to a wide interest in pharmacological agents that inhibit the activity of ABC transporters. Such agents are described in medical literature as modulators or reversal agents since they may reduce MDR in oncology (Robert and Jarry, 2003). The wide interest in these agents has resulted in the discovery of hundreds of compounds that inhibit ABC transporter activity. Some characters of these compounds involve amphiphilic properties, the presence of aromatic rings, and the positive charge at neutral pH (Zamora et al., 1988). In oncology, modulators are classified as first generation modulators (compounds already used in clinics for other indications), second generation modulators (analogues of these drugs), and third generation modulators, (drugs of original structure developed for the purpose of MDR reversal) (Robert and Jarry, 2003). Many natural toxic compounds such as plant alkaloids and flavonoids have also been described as

multidrug pumps inhibitors (Gus et al., 2001; Lania-Pietrzak et al., 2005). Isoflavones are described as potentiators of antibacterial activity of natural toxic plant compounds, indicating that they enhance activity of (endogenous) natural antimicrobial compounds (De Waard, 1997; Morel et al., 2003). Recently, the synthetic flavonoid derivative 4'-hydroxyflavone, was identified as an inhibitor of fungicide efflux in the plant pathogen *Pyrenophora tritici-repens*, capable of restoring fungicide activity against fungicide-resistant isolates back to normal wild-type sensitivity (Reimann and Deising, 2005). In view of the considerations described above, modulators of fungal ABC transporters can be regarded as promising lead compounds in disease control of plant pathogens. The putative ways these compounds can be exploited are summarized in Table 1.

In this study we selected from literature a number of modulators representing diverse chemical groups. These compounds were analyzed for their *in vitro* modulating activity of azole fungicides against isolates of *M. graminicola* varying in azole sensitivity. This fungus is regarded as the major threat of wheat crops in Europe and causes serious losses of both bread and durum wheat. Disease management is mainly based on the use of resistant wheat cultivars and chemical control, particularly with azole fungicides. Compounds which enhanced activity of the azole fungicide cyproconazole *in vitro* were further tested for their effect on accumulation of azole fungicides in mycelium and for their synergistic activities in disease control of the pathogen. Some of the modulators selected displayed disease control activity on their own. The activity of these products was further analysed.

Table 1. Putative functions of ABC transporters from plant pathogens and exploitation of modulators of these proteins in disease control

Function of ABC transporters	Effect of modulators
Prevention of accumulation of plant defence products in fungal cells during pathogenesis	Increased accumulation of plant defence products in fungal cells resulting in reduced fungal growth and colonization of plant tissue
Secretion of fungal toxins with a role in virulence	Suicidal activity on the producing fungus; reduced virulence on host plants
Reduction of sensitivity of plant pathogens to fungicides with a sensitive target site	Synergistic activity between modulators and fungicides resulting in a wider spectrum of antifungal activity of fungicide
MDR of plant pathogens	Reversal of MDR to wild-type sensitivity of multiple chemicals

Materials and methods

Fungal strains, cultural conditions and preparation of biomass suspensions

M. graminicola strains used in this study were the field isolates IPO323 and S190, originating from the Netherlands and Germany, respectively (Kema and Van Silfhout, 1997; Stergiopoulos et al., 2003a). Strains IPO323 and S190 are field isolates with a relatively high and low sensitivity to azole fungicides, respectively. Strain IPO323C1 was isolated in the laboratory upon selection for resistance to cyproconazole. The strain has a MDR phenotype to unrelated compounds such as cycloheximide and rhodamine 6G (Zwiers et al., 2002). Yeast-like cells and mycelium of these strains were grown in liquid yeast-sucrose medium (YSM) and Czapek Dox-mycological peptone (CzDMP), respectively, as described before (Zwiers et al., 2002). Cells used for inoculation experiments were harvested by centrifugation at 3,000 *g* for 10 min at 10 °C, washed once in sterile MilliQ water and resuspended in 0.15% Tween-20 at a density of 10^7 cells ml^{-1}. Cell suspensions used for accumulation experiments were washed and resuspended in 50 mM potassium phosphate buffer pH 6.0 containing 10 g L^{-1} glucose at a density of 6 mg wet weight ml^{-1}. Mycelial suspensions used for accumulation experiments were prepared by filtering cultures over a 0.85 mm pore sieve and collection on a 0.055 mm pore sieve. The collected mycelium was washed and resuspended in the same buffer as used for cell suspensions.

Toxicity bioassays

Paper discs (diameter 6 mm; Whatman) were saturated in methanolic solutions of modulators (3000 mg L^{-1}) and used in paper disc bioassays. The discs were dried, placed on plates (diameter 9 cm) with PDA (20 ml) seeded with *M. graminicola* cells (10^7 cells ml^{-1}), and amended with cyproconazole at sub-lethal concentrations. Six paper discs per modulator were used in each plate. The diameter of inhibition zones around the discs was measured after incubation at 20 °C for 14 days. The experiment was carried out in duplicate and repeated twice. In agar growth bioassays plates (diameter 9 cm) with PDA (20 ml) amended with modulators or fungicides at various concentrations were inoculated in triplicate with 5 µl drops of *M. graminicola* cell suspension ($4x10^5$

cells ml^{-1}) and incubated at 20 °C for 10 days. Then, minimal inhibitory concentrations (MICs) that fully inhibited growth were scored. The experiment was carried out in duplicate and repeated twice.

Crossed-paper strip bioassay

Filter paper strips (0.7 x 8 cm) were saturated in methanolic solutions of modulators (3000 mg L^{-1}) and cyproconazole (1 and 10 mg L^{-1}). The strips were dried and transferred to plates (diameter 9 cm) containing PDA (15 ml) and seeded with *M. graminicola* cells (10^7 cells ml^{-1}). Plates seeded with strain IPO323 contained a strip treated with 1 mg L^{-1} cyproconazole and plates with strains S190 and IPO323C1 with 10 mg L^{-1}. The growth pattern along the paper strips was visually assessed after incubation at 20 °C for 14 days.

Foliar spray experiments

Disease control activity of modulators alone and in combination with cyproconazole was tested in preventive foliar spray experiments with wheat seedlings (± 20) grown in pots (6 x 6 cm). Cultivars Obelisk and Vivant were used in experiments with strains IPO323 and S190, respectively. Foliar sprays were carried out in a spray cabinet equipped with a turn-table. Wheat seedlings (eight-day-old) were sprayed with modulators alone (0, 30, 100, 300, and 1000 mg L^{-1}) and in a mixture with cyproconazole (0.1 mg L^{-1}) for two minutes at a pressure of 0.8 bar, until run-off. Control seedlings were sprayed with 0.15% Tween-20. The seedlings were dried overnight and subsequently sprayed with cell suspensions of *M. graminicola* strains set at a density of 10^7 cells ml^{-1} in 0.1% Tween-20. Inoculated plants were placed on water-soaked cloth in sealed Perspex-lidded containers at 18 °C in climate rooms in the dark. Control plants and plants treated with modulators were placed in separate boxes in order to avoid possible vapour phase activity. After two days of incubation a 16-h-daylight period was applied. Emerging second leaves were clipped every 4-5 days to facilitate disease assessment and light penetration. Virulence was assessed visually by evaluation of necrotic leaf areas (ten leaves per treatment) and abundance of pycnidia in necrotic lesions 16 and 21 days post inoculation (dpi). An estimate of the expected interaction between cyproconazole (0.1 mg L^{-1}) and modulators (30 and 300 mg L^{-1}) was calculated

according to the equation of Colby (Colby, 1967) $E = X_p\ Y_q /100$, in which E is the expected disease as percentage-of-control by the mixture of compounds A and B (at concentrations p + q) and X_p and Y_q represent the disease as percentage-of-control with single compound A and B at concentrations p and q, respectively. A deviation of the expected response indicates synergism or antagonism. Curative foliar spray experiments were performed by application of compounds in a similar way as described above but one day after fungal inoculation. The experiments were carried out in triplicate.

Accumulation of cyproconazole

Cell and mycelial suspensions (55 ml) were incubated in flasks (300 ml) at 25 °C and 140 rpm for 30 min. At zero time [^{14}C] cyproconazole (Syngenta, Basel, Switserland) was added to an external concentration of 100 µM (1.5 MBq mmol^{-1}). Modulators were added 30 min after addition of cyproconazole to an external concentration of 100 and 300 µM. Amitriptiline, chlorpromazine, flavanone and promazine at 300 µM is equivalent to 94, 96, 47, and 106 mg L^{-1}. Cells and mycelium were harvested at intervals by vacuum filtration of samples (5 mL), washed five times with 5 ml of phosphate buffer pH 6.0 and radioactivity in the biomass was measured with a Beckman LS6000TA liquid scintillation counter. Accumulation of [^{14}C] cyproconazole was calculated as nmol mg^{-1} dry weight of biomass (Zwiers et al., 2002).

Results

Selection of experimental modulators

In a classical overview on drugs that alter MDR in cancer cells, Ford and Hait (1990) categorized modulators as calcium channel blockers, calmodulin antagonists, Vinca alkaloids, steroids, hormonal analogs, and miscellaneous hydrophobic cationic compounds (Table 2). Since that time numerous other modulators have been described in literature and a number of them are also listed in Table 2. Modulators used in the present study are marked in bold. Calcium channel blockers and cyclosporins were not included since similar experiments with *B. cinerea* were not successful (Hayashi et al., 2003; unpublished results). A relatively large number of phenothiazines were selected

Table 2. List of compounds described in literature as modulator of drug efflux from cancer cells. Compounds in bold were used in the present study

Category	Examples of compounds	Pharmacological properties
Calcium channel blockers (Ford and Hait, 1990)	Verapamil, nifedipine, and related compounds from different structural classes	Coronary vasodilator
Calmodulin antagonists (Ford and Hait, 1990; Kolaczkowski et al., 2003)	Phenothiazines like **chlorpromazine, promazine**, and **thioridazine**	Antipsychotic drug
Alkaloids (Ford and Hait, 1990; Pizzolato and Saltz, 2003)	**Camptothecin**, vinblastine, vincristine and related products	Cytotoxic activity
Steroids and hormonal analogs (Ford and Hait, 1990; Sparreboom et al., 2003)	Progesteron, **diethylstilbestrol**	Hormonal activity
Miscellaneous hydrophobic cationic compounds (Ford and Hait, 1990)	**Quinidine** **Reserpine**	Antiarrhytmic activity
Natural polyphenols and synthetic derivatives (Conseil et al., 2000; Hooijberg et al., 1997; Vaidyanathan and Walle, 2003; Wu et al., 2005)	**Chrysin**, **epicathechin, genistein, naringenin, quercetine, resorcinol, rutin**	Secondary plant metabolites; important constituents of human daily food
Flavonoids (Lania-Pietrzak et al., 2005)	**Flavanone**, flavone, flavonol, isoflavones and flavolignan	Secondary plant metabolites; important constituents of human daily food
Cyclosporin derivatives (Sparreboom et al., 2003)	Cyclosporin A	Immunosuppression activity
Rifamycin derivatives (Courtois et al., 1999; Fardel et al., 1995)	**Rifamycin B**	antibacterial drug
Opioids (Wandel et al., 2002)	**Loperamide**	Antidiarrheal drug
Tricyclic serotonin re-uptake inhibitors (Varga et al., 1996)	**Amitryptiline, imapramin**	Antipsychotic drug

because some of these compounds showed a strong synergistic activity with the azole fungicide oxpoconazole against *B. cinerea in vitro* (Hayashi et al., 2003). Most of the polyphenol and flavonoid modulators listed are naturally products that abundantly occur in plants, including food crop species. This suggests that mammalian toxicity of these compounds is low. A modulating activity of fungal ABC transporters by plant

polyphenols and flavonoids may implicate that they can enhance the activity of the plants' own natural antimicrobial compounds. Such a mechanism might contribute to the basal insensitivity of non-host plant to plant pathogens. In order to test this hypothesis polyphenol and flavonoid compounds were tested in relatively large numbers. The flavonoid 2-(4-ethoxy-phenyl)-chromen-4-one was described as an azole-efflux inhibitor of *Pyrenophora tritici-repentis* (Reimann and Deising, 2005). Since this compound is not commercially available we tested the structural analogue, 5,7-dimethoxy-2-phenyl-chromen-4-one.

Interaction between cyproconazole and modulators in paper disc bioassays

Experimental modulators with fungitoxic activity to most of the *M. graminicola* strains tested were amitriptyline, the phenothiazines chlorpromazine, promazine and thioridazine, diethylstilbestrol and flavanone (Table 3). Phenothiazines had a relatively high fungitoxic activity. The activity of amitriptyline seemed to be weaker to strain S190 as compared to wild-type strain IPO323. For most of these compounds inhibition zones in plates with cyproconazole were larger than in plates without the fungicide, suggesting that the compounds did potentiate cyproconazole activity. The phenothiazines had a relatively strong effect against all *M. graminicola* strains tested, suggesting that these are interesting candidate compounds for further research. Experimental modulators without fungitoxic activity under the test conditions used included plant alkaloids, polyphenols and flavonoids (Table 3). None of these compounds potentiated the activity of cyproconazole except for imipramine and loperamide in tests with strain IPO323.

Interaction between cyproconazole and modulators in crossed-paper strip bioassays

All compounds were screened for modulating activity of cyproconazole in crossed-paper strip bioassays with *M. graminicola* IPO323, S190 and IPO323C1. The phenothiazines chlorpromazine, promazine and thioridazine clearly synergized the activity of cyproconazole. The modulating activity of these compounds with strain IPO323 is shown in Fig. 1. Diethylstilbestrol, flavanone, loperamide, naringenin, and quinidine showed a weak synergistic interaction with cyproconazole for growth of strain

IPO323C1 only. All other interactions tested were independent except for resorcinol that antagonized the activity of cyproconazole to all strains tested, especially strain S190.

Table 3. Activity of putative modulators of ABC transporter activity in paper disc bioassays in the absence and presence of cyproconazole on growth of *Mycosphaerella graminicola* strains IPO323 (wild-type), IPO323C1 (cyproconazole resistant laboratory mutant), and S190 (field isolate with relatively low cyproconazole sensitivity). Numbers represent zones of growth inhibition (mm) around discs impregnated with modulators (3000 mg L^{-1}). Cyproconazole concentration in agar plates seeded with IPO323 was 0.001 mg L^{-1} and in plates with IPO323C1 and S190 0.01 mg L^{-1}

Modulator	Strain IPO323		Strain IPO323C1		Strain S190	
	- Cyproconazole	+ Cyproconazole	- Cyproconazole	+ Cyproconazole	- Cyproconazole	+ Cyproconazole
Control	0	0	0	0	0	0
Amitriptyline	2.2 ± 0.7*	2.2 ± 0.7	1.0 ± 0.6	2.0 ± 0.6*	0	1.4 ± 1.4*
Camptothecin	0	0	0	0	0	0
Chlorpromazine	4.3 ± 1.1	5.3 ± 0.4*	5.0 ± 1.2	8.5 ± 2.7*	3.8 ± 0.4	5.3 ± 0.4*
Chrysin	0	0	0	0	0	0
Cyclosporin	0	0	0	0	0	0
Diethylstilbestrol	1.4 ± 0.5	2.2 ± 0.4*	0	2.8 ± 0.4*	1.0 ± 0.6	1.6 ± 0.8
Epicathechin	0	0	0	0	0	0
Flavanone	1.0 ± 0	2.0 ± 0*	1.4 ± 0.5	2.0 ± 0.6*	2.2 ± 0.4	3.4 ± 0.5*
Imipramine	0	0.5 ± 0.5	0	0	0	0
Loperamide	0	0.3 ± 0.4	0	0	0	0
Naringenin	0	0	0	0	0	0
Promazine	1.8 ± 1.1	4.0 ± 1.2*	1.8 ± 0.4	2.3 ± 0.4	0.8 ± 0.4	2.0 ± 0*
Quercetin	0	0	0	0	0	0
Quinidine	0	0	0	0	0	0
Reserpine	0	0	0	0	0	0
Resorcinol	0	0	0	0	0	0
Rutin	0	0	0	0	0	0
Thioridazine	4.5 ± 0.5	6.5 ± 0.5*	4.0 ± 0.7	7.3 ± 0.8*	5.8 ± 0.8	6.5 ± 0.5
Verapamil	0	0	0	0	0	0
5,7-Dimethoxy-2-chromen-4-one	0	0	0	0	0	0

* Mean values of inhibition zone (± standard deviation) on plates with and without cyproconazole differ significantly ($P = 0.05$)

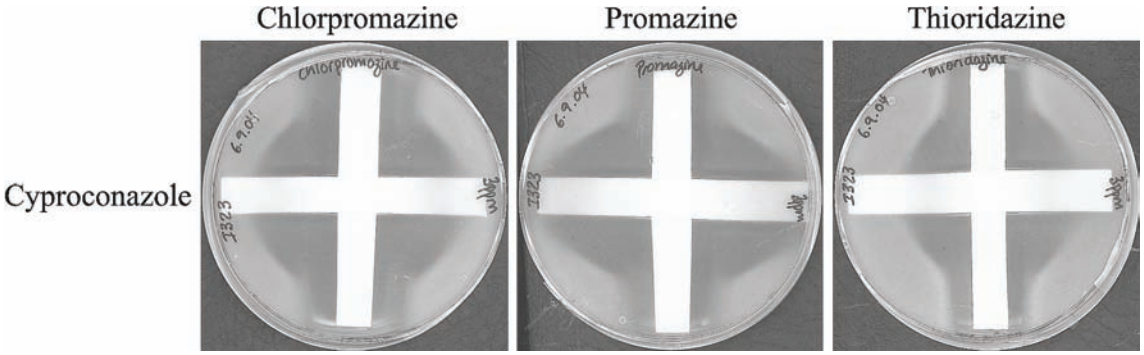

Fig. 1. Synergistic activity between cyproconazole and the putative modulators of ABC transporter activity chlorpromazine (left panel), promazine (middle panel), and thioridazine (right panel) to growth of *Mycosphaerella graminicola* strain IPO323 in crossed-paper strip experiments. The horizontal strips were impregnated with cyproconazole (1 mg L^{-1} in methanol) and the vertical strips with modulators (3000 mg L^{-1} in methanol).

MIC values of modulators in agar growth tests

The MIC values of eight experimental modulators with synergistic activity in mixtures with cyproconazole in paper disc bioassays were determined in agar growth tests. Rutin, which did not display any synergism and cyproconazole itself were included as controls. Results presented in Table 4 indicate that the toxicity of the modulators towards the *M. graminicola* IPO323 and S190 is low as compared to that of cyproconazole. For strain IPO323, the ratio between the MIC value of modulator and cyproconazole amounts to 1.000 or even higher for most of the modulators tested. Only for diethylstilbestrol a relatively low value was found (ratio 100). The MIC values of two out of three phenothiazines tested (promazine, thioridazine) correlated with the MIC values of cyproconazole for strain IPO323 and S190.

Disease control activity of modulators

Disease control activity of some of the experimental modulators listed in Table 4 was tested against *M. graminicola* strains IPO323 and S190 on wheat seedlings of cultivars Obelisk and Vivant, respectively, in preventive foliar spray experiments. In control treatments, the first symptoms became visible at 8 dpi as small chlorotic spots near the tip of the leaves. In time, the lesions expanded longitudinally across the leaves and developed into necrotic lesions covered with pycnidia. At 21 dpi, the percentage of leaf area with these symptoms on cultivars Obelisk and Vivant were 64 and 87,

respectively. The disease control activity of eight modulators was studied (Table 5). All compounds showed significant disease control activity in treatments with 30 and 300 mg L^{-1} (P<0.05). Promazine was the most active compound against strain IPO323 on wheat cultivar Obelisk since disease control activity amounted 97% of the control. Disease control activity for the other compounds varied from 23 to 85% (Table 5A). Disease control activity of the modulators against strain S190 on wheat cultivar Vivant was also obvious. Amitriptyline and quinidine were the most active compounds, showing disease control activity up to 78% (Table 5B). Treatment of plants with modulators at a concentration of 1,000 mg L^{-1} or higher caused phytotoxic symptoms, visible as necrotic lesions at 10 dpi (results not shown). Visible necrotic symptoms were not observed at 300 mg L^{-1} or lower.

Experimental modulators with a relatively high disease control activity in the preventive foliar spray tests (amitriptyline, loperamide, and promazine) were studied further in curative foliar spray tests by applying foliar sprays with the compounds one day after inoculation of the wheat seedlings. The concentration range tested was relatively low and disease development was assessed at 16 and 21 dpi (Fig. 2). The data in this figure illustrate that all compounds tested have a significant effect on disease development, especially at 16 dpi. At 21 dpi, disease control activity is still obvious but lower as compared to 16 dpi, indicating that the activity of modulators is transient in time.

Table 4. Minimal inhibitory concentration (MIC) of modulators and cyproconazole to growth of *Mycosphaerella graminicola* strains IPO323 and S190 in agar growth tests

Compounds	MIC strain IPO323	MIC strain S190
Modulators		
Amitriptyline	>300*	>300
Chlorpromazine	300	300
Diethylstilbestrol	30	30
Flavanone	300	300
Loperamide	>300	>300
Promazine	300	>300
Quinidine	>300	>300
Rutin	>300	>300
Thioridazine	100	300
Azole fungicide		
Cyproconazole	0.3	1.0

* mg L^{-1}

Table 5. Activity of compounds described in literature as modulators of ABC transporters in control of *Mycosphaerella graminicola* on wheat seedlings in preventive foliar spray experiments

(A) Control of *M. graminicola* IPO323 on wheat seedlings of cultivar Obelisk

Modulator	Necrotic area of leaves treated with modulators as percentage of control treatment*		Disease control of modulators as percentage of control	
	30 mg L^{-1}	300 mg L^{-1}	30 mg L^{-1}	300 mg L^{-1}
Amitriptyline	34.1 ± 8.9	62.2 ± 5.2	65.9	37.8
Chlorpromazine	59.3 ± 4.4	43.7 ± 5.2	40.7	56.3
Diethylstilbestrol	32.6 ± 10.0	23.7 ± 8.1	67.4	76.3
Flavanone	62.2 ± 11.8	63.7 ± 3.7	37.8	36.3
Loperamide	46.7 ± 5.2	14.1 ± 8.9	53.3	85.9
Promazine	15.6 ± 5.2	3.0 ± 2.2	84.4	97.0
Quinidine	76.3 ± 8.1	77.8 ± 4.4	23.7	22.8
Thioridazine	31.1 ± 8.1	17.0 ± 4.4	68.9	83.0

* Necrotic area (± standard deviation) in control was 64.0 ± 6.6 (set at 100%)

(B) Control of *M. graminicola* S190 on wheat seedlings of cultivar Vivant

Modulator	Necrotic area of leaves treated with modulators as percentage of control treatment*		Disease control of modulators as percentage of control	
	30 mg L^{-1}	300 mg L^{-1}	30 mg L^{-1}	300 mg L^{-1}
Amitriptyline	31.0 ± 9.5	24.3 ± 5.2	69.0	75.7
Chlorpromazine	62.0 ± 11.2	24.0 ± 7.2	38.0	76.0
Diethylstilbestrol	73.0 ± 15.3	47.9 ± 5.4	27.0	52.1
Flavanone	52.5 ± 11.0	36.3 ± 8.1	47.5	63.7
Loperamide	51.1 ± 4.6	58.2 ± 8.0	48.9	41.8
Promazine	44.3 ± 7.5	27.8 ± 3.3	55.7	72.2
Quinidine	38.9 ± 7.4	21.6 ± 5.1	61.1	78.4
Thioridazine	49.3 ± 4.3	68.0 ± 11.6	50.7	32.0

* Necrotic area in control (± standard deviation) was 87.1 ± 13.2 (set at 100%)

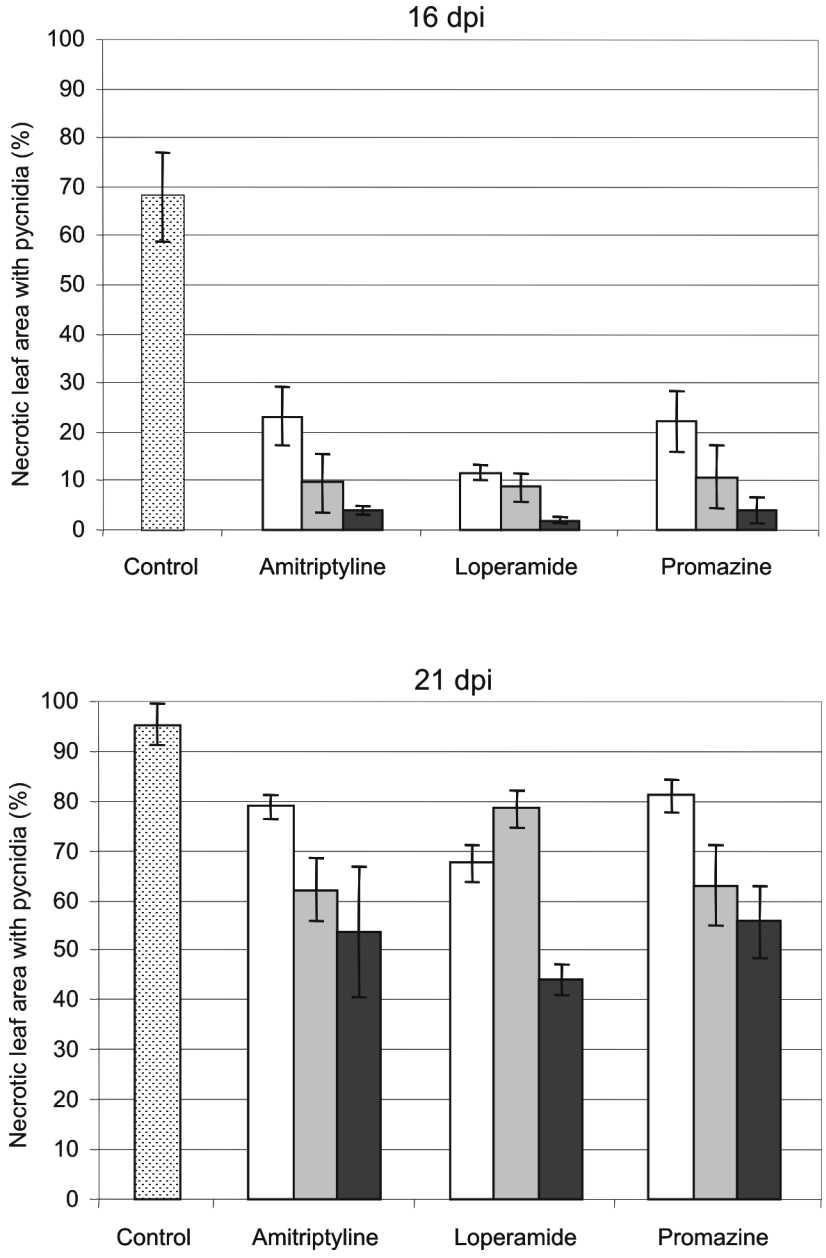

Fig. 2. Activity of amitriptiline, loperamide, and promazine in control of *Mycosphaerella graminicola* IPO323 on wheat seedlings of cultivar Obelisk in curative foliar spray tests. Concentrations of compounds tested were 1 (white bars), 10 (gray bars), and 100 mg L^{-1} (black bars). Control (dotted bar). Disease was assessed at 16 (top panel) and 21dpi (bottom panel). Bars represent mean percentage of necrotic leaf area with pycnidia and standard deviation.

Disease control activity of mixtures of modulators and cyproconazole

Interactions between experimental modulators and cyproconazole in disease control were studied in mixtures of modulators at 30 and 300 mg L^{-1}. The cyproconazole concentration in the mixtures was set at 0.1 mg L^{-1} since preliminary experiments demonstrated that this rate controlled disease in foliar spray experiments for about 50%, which is the optimal percentage to study synergism with other compounds (Table 6). The experiments were performed simultaneously with the experiments to assess the disease control activity of modulators in preventive foliar spray experiments (Table 5). An overview of the observed and expected necrotic leaf areas in disease control experiments of *M. graminicola* strains IPO323 and S190 on wheat cultivars Obelisk and Vivant indicates that observed necrotic areas of leaves treated with mixtures are similar or larger than the expected necrotic leaf areas, suggesting that synergistic activities in interactions tested are absent (Table 6).

Effect of modulators on accumulation of cyproconazole

Accumulation of cyproconazole in the absence of experimental modulators in both yeast-like cells and mycelium of *M. graminicola* IPO323 and S190 was slightly transient in time (Fig. 3). The levels of accumulation by strain S190 were higher as compared to strain IPO323. Addition of all modulators tested caused an instantaneous increase in cyproconazole accumulation. For most modulators the increase in fungicide accumulation was transient except for chlorpromazine added to yeast-like cells. In this case, the accumulation level of cyproconazole remained at an almost constant elevated level. Modulators added to mycelial and cell suspensions at a final concentration of 100 µM caused similar effects on accumulation of cyproconazole but were less pronounced (results not shown). Loperamide, quinidine and thioridazine (300 µM) did not significantly enhance accumulation of cyproconazole in both cells and mycelium (results not shown).

Table 6. Interactions between cyproconazole and modulators in disease control of *Mycosphaerella graminicola* on wheat seedlings[a]

Modulator in mixture with cyproconazole (0.1 mg L^{-1})[b]	Observed and expected necrotic leaf area of wheat seedlings sprayed with mixtures of cyproconazole and modulators			
	(A) Cultivar Obelisk inoculated with *M. graminicola* IPO323		(B) Cultivar Vivant inoculated with *M. graminicola* S190	
	Observed necrotic leaf area[c]	Expected necrotic leaf area[d]	Observed necrotic leaf area	Expected necrotic leaf area
Amitriptyline (30 mg L^{-1})	68.5 ± 5.2	14.7	28.1 ± 9.4	18.3
Amitriptyline (300 mg L^{-1})	32.6 ± 5.3	26.7	16.6 ± 5.6	14.4
Chlorpromazine (30 mg L^{-1})	50.2 ± 5.0	25.4	43.4 ± 9.0	36.6
Chlorpromazine (300 mg L^{-1})	20.6 ± 8.9	18.8	24.9 ± 14.0	14.2
Diethylstilbestrol (30 mg L^{-1})	34.0 ± 2.2	13.8	36.1 ± 9.6	43.1
Diethylstilbestrol (300 mg L^{-1})	18.7 ± 6.7	10.2	62.6 ± 9.4	28.2
Flavanone (30 mg L^{-1})	59.2 ±10.0	26.7	31.5 ± 7.7	31.0
Flavanone (300 mg L^{-1})	50.0 ± 11.8	27.4	22.8 ± 4.2	21.4
Loperamide (30 mg L^{-1})	17.0 ± 3.7	20.1	51.5 ± 6.3	30.1
Loperamide (300 mg L^{-1})	20.1 ± 5.2	6.1	38.3 ± 4.1	34.3
Promazine (30 mg L^{-1})	3.0 ± 3.0	6.7	25.1 ± 5.2	26.1
Promazine (300 mg L^{-1})	11.0 ± 5.2	1.3	20.7 ± 3.4	16.4
Quinidine (30 mg L^{-1})	70.0 ± 8.8	32.8	34.1 ± 4.7	23.0
Quinidine (300 mg L^{-1})	62.0 ± 8.0	33.4	13.4 ± 5.6	12.7
Thioridazine (30 mg L^{-1})	27.7 ± 7.0	13.3	45.9 ± 2.7	29.1
Thioridazine (300 mg L^{-1})	11.0 ± 8.7	7.3	43.3 ± 6.4	40.1

[a] The results were obtained in experiments simultaneously performed with the disease control experiments with individual compounds as described in Table 5.

[b] Average of necrotic areas of leaves sprayed with cyproconazole (0.1 mg L^{-1}) as percentages of the control in experiment A and B were 43.0 ± 4.8 and 59.0 ± 8.0, respectively.

[c] Numbers represent average percentage of necrotic areas of leaves (± standard deviation) as percentage of the water controls.

[d] Expected necrotic leaf area was calculated according to Colby (1967), using values of necrotic leaf areas in single treatments of cyproconazole and modulators.

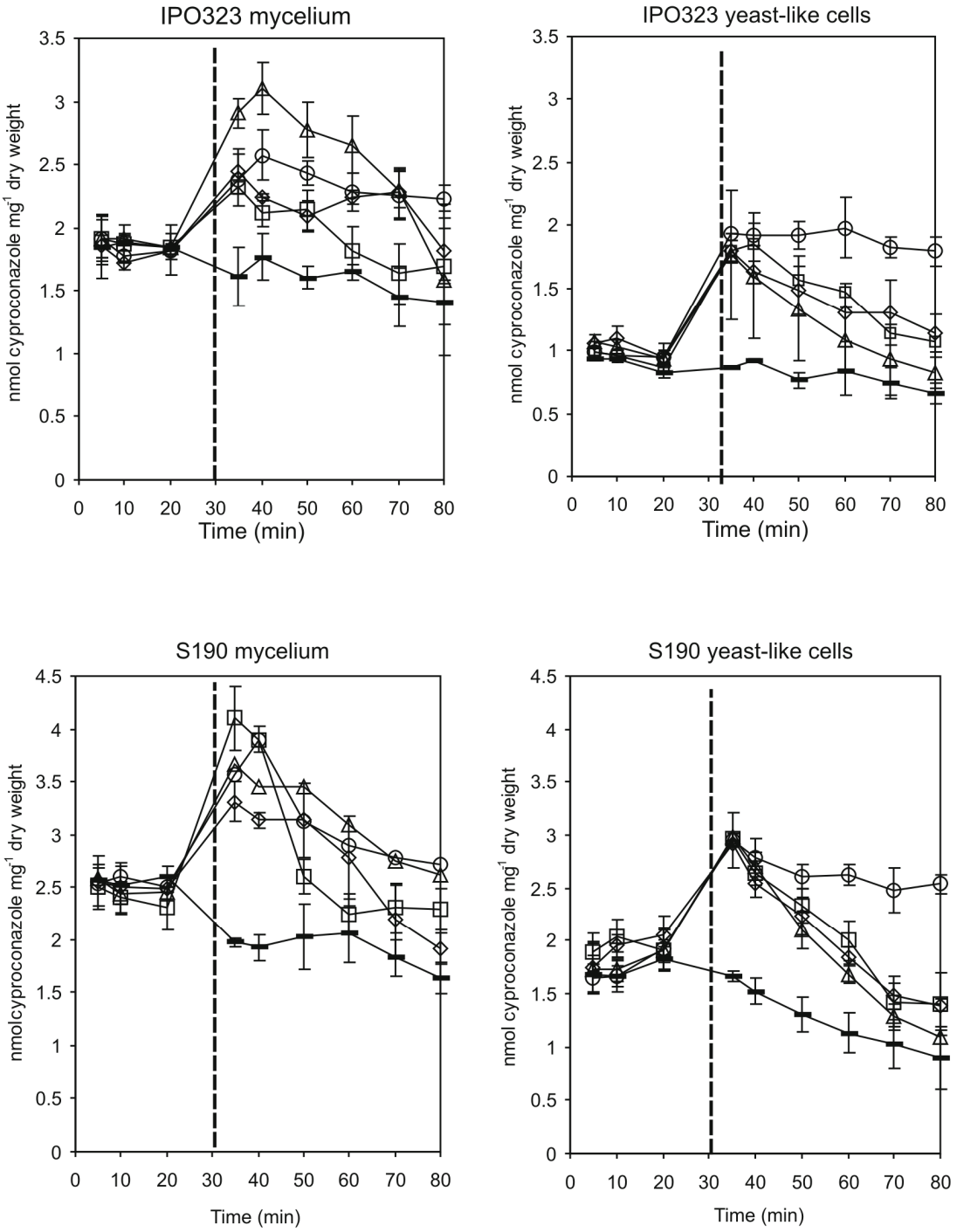

Fig. 3. Effect of modulators on accumulation of [^{14}C] cyproconazole (100 µM) by mycelium and yeast-like cells of *Mycosphaerella graminicola* IPO323 and S190. Modulators (300 µM) amitriptyline (□), chlorpromazine (○), flavanone (Δ), and promazine (◊) added 30 min after the addition of cyproconazole (dashed line). Control treatment (–). Bars indicate standard deviation of the means.

Discussion

Several modulators described in literature as compounds that alter MDR in cancer cells were also able to increase the activity of the azole fungicide cyproconazole to *M. graminicola*. This activity could be demonstrated for amitriptyline, diethylstilbestrol, flavanone and the phenothiazines chlorpromazine, promazine and thioridazine in paper disc bioassays with three strains of the pathogen that differ in sensitivity to cyproconazole. The modulating activity of the phenothiazines was also apparent in crossed-paper strip experiments. These results corroborate the synergistic activity between chlorpromazine and cyproconazole against *M. graminicola* as reported earlier (Stergiopoulos, 2003). Chlorpromazine can also modulate the activity of azole fungicides against *B. cinerea*, particularly against an azole secreting, ABC transporter BcatrD overexpression mutant (Hayashi et al., 2003). Additional studies demonstrated that amitriptyline, chlorpromazine, flavanone and promazine had an instantaneous effect on the accumulation levels of cyproconazole in yeast-like cells and mycelium of *M. graminicola*, suggesting that cyproconazole efflux activity by fungal drug transporters was inhibited. Chlorpromazine had a relatively strong effect since its reversal of efflux activity remained almost constant in time. This character of chlorpromazine may relate to its relatively strong modulating activity in the *in vitro* assays. The modulating activity may be due to affinity of the modulators to binding sites of ABC transporter proteins which results in inhibition of cyproconazole transport (Zloh et al., 2004).

Several ABC transporters of *M. graminicola* that can provide protection against azole fungicides have been described (Zwiers et al., 2003). Hence, it might be that reversal of one or more of these ABC transporters in *M. graminicola* by the phenothiazines or other compounds tested are responsible for synergism with cyproconazole. The MFS transporter MgMfs1 is also described as a potent transporter of azole fungicides (Roohparvar et al., 2006). However, the compounds tested are not described in literature as modulators of MFS transporters and therefore modulation of MgMfs1 by phenothiazines is probably not responsible for the synergism observed.

Various models have been described to explain reversal of drug efflux activity mediated by ABC transporters (Baird and Kaye, 2003). A proposed mechanism of

action is direct binding of the modulator to binding site(s) on the transporter protein which results in blocking transport in either a competitive or non-competitive mode (Zloh et al., 2004).

Foliar spray experiments with mixtures of cyproconazole and modulators demonstrated that the expected disease control activity as calculated according to Colby (Colby, 1967) was merely additive or even antagonistic. Thus, none of the modulators tested showed synergism with cyproconazole *in planta*, even not the modulators exhibiting synergism *in vitro*. This situation contrasts with the modulating activity reported for a 4'-hydroxyflavone derivative of resistance to azoles and other fungicides in *P. tritici-repens* (Reimann and Deising, 2005), but is not uncommon for MDR modulators in clinical situations (Robert and Jarry, 2003). There are several reasons that could explain the lack of *in planta* modulating activity. (1) The *in planta*-mediated degree of natural insensitivity or resistance of *M. graminicola* through ABC transporters is too low to show an interaction; (2) The mixture of fungicide and modulators may have exerted a phytotoxic or senescent effect on wheat promoting its susceptibility to the pathogen; (3) Cyproconazole is a systemic fungicide, while the modulators probably only have a residual effect on the leaf surface. These different properties may result in a rapid spatial separation of the two compounds in plant tissues and once the pathogen has invaded the host via the stomatal cavities, it is only exposed to the fungicide. For these reasons similar experiments with a MDR strain of *M. graminicola* with high levels of resistance and a systemic modulator without phytotoxicity would be recommendable. However, field isolates with such a phenotype are not available. Laboratory mutants that posses MDR phenotypes (e.g. strain IPO323C1) are impaired in virulence on wheat, and systemic modulators are not known.

All experimental modulators tested individually controlled *M. graminicola* on wheat seedlings in preventive foliar spray experiments (Table 5). A curative foliar spray test with amitriptyline, loperamide, and promazine demonstrated that at relatively low concentrations disease control is especially evident during the initial phase of disease development (Fig. 2). The disease control efficacy of the compounds may be explained in different ways. One possibility is that the presence of the modulators leads to reversal of ABC transporters that act as pathogenicity factors, such as MgAtr4 (Stergiopoulos et al., 2003b). Thus, as described in Table 1, modulator disease control activity can be

ascribed to increased accumulation of plant defense products in the pathogen or reduced secretion of fungal toxins. This may particularly apply to modulators such as amitriptyline, loperamide, and promazine which did not possess *in vitro* toxicity to *M. graminicola* in agar growth tests (Table 4). Compounds active in such a way can be regarded as disease control agents with an indirect mode of action. This hypothesis is difficult to verify since a clear role of fungal toxins and plant defense compounds in the interaction of *M. graminicola* and wheat has not been elucidated. Most of the other modulators that possessed intrinsic disease control activity have a low or moderate direct toxicity to *M. graminicola in vitro* (chlorpromazine, diethylstilbestrol, flavanone, promazine and thioridazine). This may imply that the disease control activity of these compounds may be a consequence of both modulation of ABC transporter activity and direct activity against *M. graminicola*. For instance, the MIC values of diethylstilbestrol and cyproconazole for *in vitro* growth were 30 and 0.3 mg L^{-1}, respectively, indicating a toxicity ratio between the two compounds of 100 (Table 5). If the same ratio would apply for disease control activity *in planta*, approximately 50% disease control would be expected with diethylstilbestrol at a rate of 10 mg L^{-1} (50% disease control by cyproconazole is obtained at 0.1 mg L^{-1}) (Table 6). Indeed, disease control of diethylstilbestrol is in this order of magnitude and for that reason disease control by the compound is probably due to direct toxicity to the pathogen. For compounds with a MIC value of 300 mg L^{-1} (chlorpromazine, flavanone, promazine) a similar reasoning is less obvious since their toxicity ratio with cyproconazole of 1,000 can probably not be fully explained by direct toxicity. Amitriptyline and loperamide belonged to the category of compounds with the highest MIC values determined (> 300 mg L^{-1}). Still, these compounds displayed a relatively high disease control activity, both in preventive and curative disease control tests at low concentrations. For these reasons it is likely that disease control activity by amitriptyline, loperamide and promazine is due to an indirect mode of action and not by direct toxicity.

Remarkably, modulators with the highest disease control activity against *M. graminicola* (amitriptyline, loperamide, and promazine) also potentiate the activity of cyproconazole in paper disc bioassays. As described above these two characteristics of modulators are not necessarily due to reversal of activity of the same ABC transporter(s). This reasoning would imply that the modulators inhibit activity of

multiple ABC transporters and indicates that the compounds have no reversal selectivity. Such a property would make the *in vitro* selection of new reversal agents in bioassays as described in this paper easier. The modulators tested did not display phytotoxicity at concentrations of 100 mg L^{-1} in foliar spray tests. This indicates that selective toxicity between different groups of organisms is feasible.

In conclusion, the results reported in this paper describe the first steps in the discovery and development of modulators of ABC transporter activity which potentiate the activity of azole fungicides towards plant pathogens and which may possess indirect disease control activity. Modulators with disease control activity are amitriptyline, loperamide, and promazine which are known chemical drugs for control of human diseases. Second step in the development of disease control agents could be the synthesis and screening of structural analogues of these compounds for improved biological activity, systemic activity in plants and selective activity against plant pathogens and other classes of organisms. As a third step modulators with a new chemical structure can be developed. A similar sequence of events has been described for the development of modulators for clinical use (Robert and Jarry, 2003).

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Chapter 7

General discussion

The MFS transporter MgMfs1

One aspect of this thesis involves the cloning and characterization of the *Mycosphaerella graminicola* major facilitator superfamily (MSF) transporter MgMfs1. Fungal MFS transporters facilitate the secretion of endogenously produced (host-specific) toxins. Well-known examples include the secretion of the cyclic peptide, HC-toxin, by *Cochliobolus carbonum*, the polyketide, cercosporin, by *Cercospora kikuchii*, and the cyclic sesquiterpenoid trichothecenes by *Fusarium sporotrichioides* (Alexander et al., 1999; Callahan et al., 1999; Pitkin et al., 1996). The high homology of MgMfs1 described in this thesis to several of these MFS transporters suggested a potential role for this transporter in a similar process. Although the involvement of a host-specific toxin in the *M. graminicola*–wheat pathosystem is still under debate, there are indications pointing towards the presence of such a toxin (Kema et al., 1996; Perrone et al., 1999) that has, however, not been identified. Since many fungal species belonging to the Mycosphaerellaceae are capable of producing cercosporin, it is not unlikely that such a toxin might be related to cercosporin (Goodwin et al., 2001). Indeed, MgMfs1 expression is upregulated by cercosporin and *MgMfs1* disruption mutants show an increased sensitivity to this toxin. However, as MgMfs1 does not play an apparent role in virulence (as disruption mutants were as virulent on wheat as the wild-type strain) it is concluded that the transporter is not essential for the secretion of the putative toxin, or that *M. graminicola* does not produce such a toxin. The phenotype of the disruption mutants is comparable with that observed for *BcMfs1* from *B. cinerea*, the closest *MgMfs1* homologue. *BcMfs1* is also capable of transporting cercosporin but it does not function as a virulence factor on tomato (Hayashi et al., 2002). This lack of phenotype can be explained by the fact that in all organisms sequenced to date, MFS transporters constitute a large family of members with overlapping substrate specificity. *M. graminicola* is not an exception, as *in silico* analysis of the recently sequenced genome of *M. graminicola* (http://genome.jgi-psf.org/Mycgr1/Mycgr1.home.html) shows that the pathogen contains (at least) 288 MFS transporters of which 87 are putative MFS-MDR transporters (Personal communication L.-H. Zwiers). It is obvious that the abundance of MFS transporters and the concomitant redundancy in function severely

complicates the functional analysis of these genes. The functional redundancy of the transporters can also explain why *MgMfs1* disruption mutants of *M. graminicola* display a decreased sensitivity to rhodamine 6G. The lack of MgMfs1 may be overcompensated by upregulated expression of other transporter genes with affinity for rhodamine 6G (Zwiers et al., 2003).

Although MgMfs1 does not seem to play a role in virulence, our results demonstrate that this protein is a major multidrug transporter. Until now, in filamentous fungi the research on transporters involved in MDR has focused on ATP-binding cassette (ABC) transporters. However, the exceptional broad substrate range of MgMfs1 and the degree of protection provided by this protein suggest that MFS transporters are at least as important as ABC transporters in MDR. This has implications for resistance development in fungal species. MFS transporters drive transport by means of the proton-motive force and not by the active hydrolysis of ATP as is the case for ABC transporters. As cells continuously maintain a proton motive force, transport mediated by MFS transporters may constitute a route requiring less energy to secrete toxic compounds than ABC transporters. Consequently, selection imposed by drugs might favor resistance development through MFS transporters. Our findings on the involvement of MgMfs1 in strobilurin sensitivity are in agreement with this hypothesis and will be discussed in more detail in the next section.

Strobilurin sensitivity and resistance in *M. graminicola*

Strobilurins constitute a relatively new family of broad-spectrum fungicides that are widely used for control of septoria tritici leaf blotch on wheat. Resistance to these fungicides has rapidly evolved over the past three years. Resistance is mainly the result of point mutations in the target cytochrome b gene (Gisi et al., 2000) that leads to an amino acid substitution in the encoded protein (G143A). As point mutations in target sites generally result in a very high degree of resistance, it is possible that additional mechanisms with lower levels of resistance are overlooked because their effect is masked by the dominant effect of the target-site mutation (Franz et al., 1998; White et

al., 2002). Our data indeed show that this phenomenon may occur. The data presented in chapter 3 and 4 clearly show that MgMfs1 is involved in sensitivity of *M. graminicola* to strobilurin fungicides. This is not only true *in vitro* but also *in planta* on trifloxystrobin-treated wheat seedlings. Thus, MgMfs1 could function as a determinant of baseline sensitivity to strobilurin fungicides.

The data suggest that the transporter functions as the first line of defense against these fungicides. The selection pressure imposed by application of strobilurin fungicides would not only select for mutants that already have the target site mutation (G143A) but also for strains that exhibit a higher expression of *MgMfs1* coding for a transporter with specificity for these chemicals. Because of the high number of functional redundant transporters and the high variation in expression of transporter genes between strains (Stergiopoulos et al., 2003a; Zwiers et al., 2002), it can be expected that within the population a shift towards strains overexpressing strobilurin transporters (e.g. MgMfs1) will occur. In addition, recent observations have shown that sensitive strains are still able to mate on wheat seedlings treated with a full rate of a strobilurin fungicide, indicating that sensitive strains overcome the disruption of mitochondrial respiration in the sensitive parent strain (Ware et al., 2006). If the resistance would be solely due to an alteration of the target site, this would still lead to an accumulation of the fungicides in the membrane. This could result in hampered membrane function and eventually to death of the organism. Mutations that result in increased efflux mediated by drug transporters could prevent this and safeguard normal membrane function and fitness in resistant strains with the G143A substitution in the target site.

The ABC transporter MgAtr7

The screening of ten *M. graminicola* expressed sequence tag (EST) libraries and *in silico* analysis of its genome sequence data revealed the presence of 47 ABC proteins of which 36 are true ABC transporters. Out of the 36 true ABC transporters seven belong to the pleiotropic drug resistance (PDR) class of ABC transporters that function in baseline sensitivity and resistance to azole fungicides (personal communication L.-H.

Zwiers). Previous searches for ABC transporter genes from this fungus already resulted in the identification of five of the seven PDR-type ABC transporters (Stergiopoulos et al., 2003a; Zwiers et al., 2002). In this thesis we analyzed the new gene *MgAtr7*. It encodes a novel type of ABC transporter with an extraordinary topology consisting of an ABC transporter N-terminally fused to a putative biosynthetic protein which seems to be unique for *M. graminicola* and *F. graminearum*. Functional analysis of MgAtr7 revealed that this transporter is neither involved in fungicide sensitivity nor required for virulence. However, our data suggest that MgAtr7 might function in maintenance of fungal iron homeostasis by modulating iron levels in the cell (Zwiers et al., 2006, chapter 5). Iron is an essential element for all eukaryotes and most prokaryotes, as it is a cofactor in many enzymes and a catalyst in electron transport systems. Although iron is the second most abundant metal on Earth, it is in general not easily accessible to living organisms. To mobilize iron, when present at low concentrations, most fungi produce ferric iron-specific chelators called siderophores to retrieve it from the environment. Uptake of siderophore-iron complexes is often mediated by MFS transporters but whether the secretion of siderophores is also facilitated by MFS transporters is largely unknown. However, some data suggest the involvement of ABC transporters or MFS-MDR transporters. Again the *in silico* analysis of the *M. graminicola* genome suggests that out of the 87 putative MFS-MDR transporters six are potentially involved in the transport of siderophore-iron complexes based on their homology to known siderophore transporters and / or their genomic context. Intracellular iron levels need to be tightly regulated, as iron facilitates the production of toxic reactive oxygen species (Halliwell and Gutteridge, 1984). Thus, iron homeostasis is maintained by a tight control of iron uptake, iron storage and iron transport.

Besides extracellular siderophores, most fungi have intracellular siderophores for iron storage (Haas, 2003). Our results obtained with the *MgAtr7* mutants of *M. graminicola* (chapter 5) suggest that MgAtr7 modulates the availability of iron in the cell. We hypothesize that the Dit1-PvcA moiety of MgAtr7 and the linked putative non-ribosomal peptide synthetase (NRPS) are (partly) responsible for the synthesis of a compound which is subsequently secreted by the MgAtr7 transporter moiety to the outside of the cell. This would provide *M. graminicola* with a mechanism to protect

itself against high levels of iron in the environment and alternatively to retrieve iron from an environment where iron is limiting.

Siderophores can have pleiotropic functions. In the filamentous fungus *Aspergillus nidulans*, the intracellular siderophore ferricrocin is involved in iron storage, oxidative-stress resistance, germination, and sexual development. In this fungus, ferricrocin accumulates in a highly regulated manner via transcriptional regulation of the non-ribosomal peptide synthetase SidC. Germination of conidia from *ΔsidC* mutants of *A. nidulans* was retarded under iron-depleted conditions, most likely due to reduced iron storage. Under iron-replete conditions, no difference in germination as compared to the wild-type strain was observed, indicating that the lack of FC^{-Fe} in *ΔsidC* can be compensated for by uptake of iron (Eisendle et al., 2006). In *A. nidulans*, *Neurospora crassa*, and *Penicillium chrysogenum*, iron has previously been reported to be an important factor required during conidial germination. In these fungi, germination of conidia in the absence of cytoplasmic siderophores fails or is greatly retarded in the absence of a suitable siderophore (Charlang et al., 1981; Horowitz et al., 1976). We also performed preliminary studies on the effect of iron on the *in vitro* germination of *M. graminicola* spores by determining iron-dependent growth in Grimm-Allen (GA) medium as described previously (Baakza et al., 2004). The results indicate that on this medium containing 10–100 µM of ferric iron, spore germination of knock-out mutants of *MgAtr7* is reduced compared to the control strains (Fig. 1). In contrast, no significant differences in germination frequencies were observed between the *MgAtr7* mutants and control strains both in the absence of iron and presence of high iron concentration (1 mM).

Modulators of drug transporters

As described in this thesis, drug transporters from *M. graminicola* can be involved in protection against fungicides and biotic antifungal compounds. The importance of drug transporters was described first in the medical area and the role of ABC transporters in MDR of cancer cells has led to a wide interest in pharmacological agents

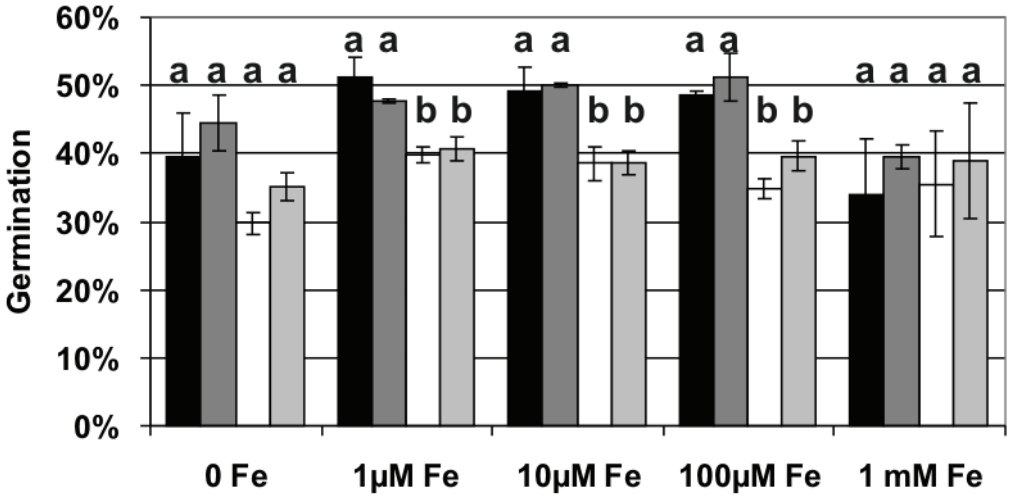

Fig. 1. Effect of ferric iron on the spore germination frequency of *Mycosphaerella graminicola* spores on Grimm-Allen (GA) medium. Germination frequency was determined 16 hours after plating 10^4 spores of two control strains; IPO323 (black bars), an IPO323 derived strain containing a disrupted copy of the ABC transporter MgAtr1 (dark grey bars), and two independent strains in which the *MgAtr7* gene was partially deleted (white and light grey bars). The bars represent average results within a single experiment performed in triplicate. Lower case letters above the bars indicate that the means do not differ significantly ($P< 0.05$, Student's *t*-test).

that inhibit the activity of ABC transporters. Such agents are described in medical literature as blockers, modulators or reversal agents (Robert and Jarry, 2003). Extensive searches have resulted in the discovery of hundreds of compounds that inhibit ABC transporter activity. Many of these inhibitors are natural toxic compounds such as plant alkaloids and flavonoids (Gus et al., 2001; Lania-Pietrzak et al., 2005).

Similarly, compounds could be developed that inhibit the activity of drug transporters in plant pathogens and thus annihilate MDR or even antagonize the activity of transporters involved in fungal virulence. Consequently, such compounds would act as synergists of agricultural fungicides. This approach could be validated in experiments with the plant pathogen *Pyrenophora tritici-repentis*. In this fungus, the synthetic flavonoid derivative 4'-hydroxyflavone, functioned as an inhibitor of fungicide efflux, thereby restoring fungicide sensitivity in a fungicide-resistant strain (Reimann and Deising, 2005). In chapter 6, a number of modulators representing diverse chemical groups were selected from literature, and analyzed for their capacity to modulate the

activity of azole fungicides against strains of *M. graminicola in vitro*. Indeed several modulators that alter MDR in cancer cells (amitriptyline, diethylstilbestrol, flavanone and the phenothiazines chlorpromazine, promazine and thioridazine) were also able to increase the activity of the azole fungicide cyproconazole to three strains of *M. graminicola* with different cyproconazole sensitivity. These results corroborate the synergistic activity between chlorpromazine and cyproconazole against *M. graminicola* as reported before (Stergiopoulos and De Waard, 2002). Chlorpromazine is also a potent modulator of azole secretion in a mutant of *B. cinerea* that overexpresses the ABC transporter BcatrD (Hayashi et al., 2003). Since chlorpromazine is a medical drug, it can not be used as a modulator in agricultural practice. However, it may be useful as a lead product for the development of other compounds with commercial potential.

Some of the modulators instantaneously affected the accumulation levels of cyproconazole in *M. graminicola*, suggesting that cyproconazole efflux activity by fungal drug transporters was inhibited. Several ABC transporters of *M. graminicola* have been described that can provide protection against azole fungicides (Zwiers et al., 2003). Hence, it might be that inhibition of one or more of these ABC transporters by phenothiazines, or other compounds tested, is responsible for synergism with cyproconazole and the observed increased accumulation. Various models have been described to explain reversal of drug resistance mediated by ABC transporters (Baird and Kaye, 2003). Direct binding of the modulator to binding site(s) on the transporter protein could result in blocking transport of cyproconazole. This inhibition could be either competitive or non-competitive (Zloh et al., 2004).

This thesis demonstrates that besides ABC transporters, also the MFS transporter, MgMfs1, is a potent transporter of azole fungicides (Roohparvar et al., 2006a). None of the modulators of ABC transporters tested have been described in literature as modulators of MFS transporters, and therefore it is unlikely that modulation of MgMfs1 is responsible for the synergism observed. However, our results indicate that a search for MFS transporter modulators would also be worthwhile.

Curative foliar spray tests using amitriptyline, loperamide, and promazine demonstrated that these compounds not only act as *in vitro* modulators of fungicide activity but also exhibit disease control activity of *M. graminicola in planta* (Roohparvar et al., 2006b). These two effects are not necessarily due to inhibition of the

same ABC transporter(s). Probably, modulators inhibit activity of multiple ABC transporters. The modulators tested did not display phytotoxicity at concentrations of 100 mg L^{-1} in foliar spray tests indicating that selective toxicity between different groups of organisms exists.

The observed efficacy of the compounds as indirect disease control agents could potentially be based on inhibition of ABC transporters that secrete pathogenicity factors, such as MgAtr4 (Stergiopoulos et al., 2003b). This may particularly apply to modulators without *in vitro* toxicity to *M. graminicola*. Modulators with this type of activity may function as lead compounds in the discovery of a new class of modern disease control agents.

Chapter 6 describes the first steps in the discovery and development of modulators of ABC transporter activity which potentiate the activity of azole fungicides towards plant pathogens and which may possess an indirect mode of action. The modulators that showed disease control activity, amitriptyline, loperamide, and promazine, are well known medical drugs for control of human diseases. Screening of structural analogues of the latter compounds for improved biological activity *in planta* and selective activity between plant pathogens and other classes of organisms could initiate the development of agrochemicals with indirect disease control activity. As a next step modulators with a new chemical structure can be designed and tested. A similar sequence of events has been described for the development of modulators for clinical use (Robert and Jarry, 2003).

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Summary

Wheat is the most important cereal crop in the world. It occupies 17% of the cultivated land and is the main source of food for 35% of the world population. In Iran, wheat is the most important agricultural crop and bread as its main product provides over 50% of daily consumed calories. The ascomycetous fungus *Mycosphaerella graminicola* (Fuckel) J. Schroeter is the causal agent of septoria tritici leaf blotch disease of wheat. The fungus has gradually emerged as one of the most damaging foliar pathogens especially in areas with high rainfall during the growing season. Septoria tritici leaf blotch disease is mainly managed by using resistant cultivars, cultural practices, and chemical control. Extensive application of different classes of fungicides to control the disease has imposed selection pressure on this pathogen leading to evolution of fungicide-resistant strains.

The objective of this thesis was the analysis of major facilitator superfamily (MFS) and ATP-binding cassette (ABC) drug efflux transporters of *M. graminicola* for their role in fungicide sensitivity, multidrug resistance (MDR) development and virulence on wheat. The thesis also describes a novel type of ABC transporter with a role in iron homeostasis. Inhibitors of drug transporters that function as virulence factors may act as indirect disease control agents and therefore, the potency of medical modulators of ABC drug transporters to control septoria leaf blotch on wheat seedlings was investigated.

Chapter 1 presents a general overview on the global importance of wheat, septoria leaf blotch, *M. graminicola*, and disease management. Special attention is given to chemical control of the disease, fungicide resistance, and the role of drug transporters in fungicide sensitivity and resistance.

Chapter 2 gives a review on MFS transporters with special emphasis on drug transporters and their relevance in plant pathogenic fungi. This chapter provides an overview of common characteristics of MFS transporters, including their classification, physical characteristics, and the mechanisms of transport. Furthermore, the role of several MFS transporters from plant pathogenic fungi in secretion of various types of antifungal compounds, MDR and virulence are reviewed. Finally, the impact of fungal

MFS drug transporters on fungicide baseline sensitivity, MDR, and drug discovery is discussed.

The **chapters 3** and **4** focus on the identification and functional analysis of the MFS transporter gene *MgMfs1*, the first MFS transporter gene cloned from *M. graminicola* with high homology to fungal MFS transporters within the DHA2 family, involved in mycotoxin secretion and MDR. The results described in **chapter 3** indicate that MgMfs1 is not involved in virulence on wheat. However, heterologous expression of this gene in yeast and the phenotypic characterization of *MgMfs1* disruption mutants of *M. graminicola* demonstrate that *in vitro* MgMfs1 can act as a very potent multidrug transporter, capable to transport a wide range of substrates such as fungal toxins, plant metabolites, and fungicides, particularly strobilurins. In **Chapter 4**, it is demonstrated that MgMfs1 plays a significant role in both *in vitro* and *in planta* sensitivity of *M. graminicola* to the strobilurin fungicide trifloxystrobin. The efflux of strobilurin fungicides in *MgMfs1* disruption mutants is reduced, and in disease control experiments on wheat seedlings these disruption mutants exhibit an increased sensitivity to trifloxystrobin. **Chapter 4** also demonstrates that, besides the dominant G143A target site mutation in the cytochrome b gene, overexpression of *MgMfs1* may act as an additional protection mechanism in strobilurin-resistant field strains. *MgMfs1* overexpression is probably required for proper maintenance of membrane functioning and normal fitness of these strains in the presence of strobilurins.

Chapter 5 describes the molecular cloning and functional characterization of *MgAtr7*, an ABC transporter gene with high homology to fungal ABC transporters involved in azole sensitivity that seems to be unique for *M. graminicola* and *Fusarium graminearum*. The encoded protein MgAtr7 is a novel hybrid type of ABC transporter with the $[NBF\text{-}TM]_2$ configuration which contains a motif characteristic for a dityrosine / pyoverdine biosynthesis protein at the N-terminus, and is the first member of a new class of fungal ABC transporters harboring both a transporter and a biosynthetic moiety. Functional analyses revealed that the gene is involved neither in fungicide sensitivity nor in virulence on wheat, but functions in maintenance of iron homeostasis.

Chapter 6 describes the ability of medical drugs known to modulate the activity of ABC transporters, to potentiate the activity of the azole fungicide cyproconazole against *in vitro* growth of *M. graminicola* and to control disease development of the pathogen

on wheat seedlings. It is shown that some of these compounds can synergize cyproconazole activity *in vitro* and inhibit efflux activity of the fungicide from fungal cells. However, synergistic interactions between the modulators and cyproconazole were not observed *in planta*. Some of the compounds have virtually no toxic activity to *M. graminicola in vitro*, but do show a significant disease control activity on wheat seedlings in preventive and curative foliar spray experiments. The results suggest that these compounds have an indirect disease control activity based on modulation of fungal ABC transporters essential for virulence. Such modulators may constitute a new class of disease control agents.

Chapter 7 represents the general discussion of the thesis with special emphasis on selected topics, such as the exceptional broad substrate specificity of the multidrug transporter MgMfs1, the role of the transporter in strobilurin sensitivity and resistance of *M. graminicola*, the function of the ABC transporter MgAtr7 in iron-siderophore transport, and the potential of modulators of ABC transporters to act as lead products in the development of disease control agents.

In conclusion, the data presented in this thesis show that the MFS drug transporter MgMfs1 from *M. graminicola* plays an important role in fungicide sensitivity and resistance, specifically in relation to the strobilurin fungicides. A new type of ABC drug transporter with a new function in iron homeostasis was discovered. In addition, we demonstrated that medical drugs known as modulators of ABC transporters can have an indirect disease control activity and may constitute a new class of disease control agents.

Samenvatting

Tarwe is wereldwijd het belangrijkste graangewas. Het bestrijkt 17% van het landbouwareaal en is de voornaamste voedselbron voor 35% van de wereldbevolking. Ook in Iran, waar brood voorziet in meer dan 50% van de dagelijkse voedselbehoefte, is tarwe is het belangrijkste landbouwgewas.

De schimmel *Mycosphaerella graminicola* (Fuckel) J. Schröt is de veroorzaker van de septoria bladvlekkenziekte van tarwe. Deze schimmel heeft zich geleidelijk ontwikkeld tot één van de meest schadelijke bladpathogenen, speciaal in gebieden met veel regen tijdens het groeiseizoen. De bestrijding van de septoria bladvlekkenziekte is vooral gebaseerd op resistentieveredeling, cultuurmaatregelen en het gebruik van fungiciden. De hoge selectiedruk op *M. graminicola* door intensief gebruik van diverse klassen van fungiciden heeft geleid tot de ontwikkeling van fungicide-resistente stammen van deze schimmel.

Het doel van het onderzoek beschreven in dit proefschrift betreft de analyse van de rol die Major Facilitator Superfamily (MFS) en ATP-binding cassette (ABC) drug efflux transporters van *M. graminicola* spelen bij de gevoeligheid voor fungiciden, de ontwikkeling van (multi)drug resistentie (MDR), en virulentie op de waardplant tarwe. Er wordt een nieuw type ABC transporter beschreven met een rol in ijzer metabolisme. Remmers (modulatoren) van ABC transporters die als virulentiefactor fungeren zouden ingezet kunnen worden als indirect werkend middelen tegen plantenziekten. Daarom werd de werking van diverse in de medische literatuur beschreven modulatoren onderzocht op activiteit tegen de septoria bladvlekkenziekte op tarwezaailingen.

Hoofdstuk 1 geeft een overzicht van de betekenis van tarwe, de septoria bladvlekkenziekte, *M. graminicola*, en bestrijdingsmaatregelen tegen deze ziekte. Speciale aandacht wordt besteed aan de chemische bestrijding van de ziekte, het mogelijk mechanisme van gevoeligheid en resistentie tegen fungiciden en de rol hierbij van drug transporters.

Hoofdstuk 2 geeft een overzicht van MFS transporters met speciale aandacht voor drug transporters en hun belang voor plantenpathogene schimmels. In dit hoofdstuk worden de algemene kenmerken van MFS transporters beschreven zoals hun

klassificatie, fysische eigenschappen en transportmechanisme. Verder wordt in dit hoofdstuk de rol van verschillende MFS transporters voor plantenpathogene schimmels bij de secretie van fungitoxische verbindingen, MDR en virulentie beschreven. Ten slotte wordt nagegaan in hoeverre MFS transporters als target kunnen fungeren bij de ontwikkeling van nieuwe bestrijdingsmiddelen.

De **hoofdstukken 3** en **4** handelen over de identificatie en functionele analyse van *MgMfs1*. Dit is het eerste MFS transporter-coderende gen dat gekloneerd is uit *M. graminicola*. Het vertoont grote homologie met schimmel MFS transporters van de DHA2 familie, die betrokken is bij de secretie van mycotoxinen en MDR. De resultaten beschreven in **hoofdstuk 3** geven aan dat MgMfs1 niet betrokken is bij virulentie op tarwe. Heterologe expressie van dit gen in bakkersgist en de fenotypische karakterisering van *M. graminicola MgMfs1* disruptanten laten echter zien dat MgMfs1 *in vitro* functioneert als een zeer krachtige multidrug transporter die affiniteit heeft voor een verscheidenheid aan substraten, zoals schimmeltoxinen, plantenmetabolieten en fungiciden (in het bijzonder strobilurinen). In **hoofdstuk 4** wordt vervolgens aangetoond dat MgMfs1 zowel *in vitro* als *in planta* een rol speelt bij de gevoeligheid van *M. graminicola* voor het strobilurine fungicide trifloxystrobine. In *MgMfs1* disruptanten is de efflux van dit strobilurine fungicide verlaagd en in spuitproeven op tarwezaailingen vertonen de mutanten een hogere gevoeligheid voor dit middel dan wild-type isolaten. De resultaten beschreven in **hoofdstuk 4** laten ook zien dat strobilurine-resistente veldisolaten met een G143A mutatie in cytochroom b (de aangrijpingsplaats van strobilurinen) overexpressie van *MgMfs1* vertonen. Dit zou kunnen wijzen op een beschermingsmechanisme tegen accumulatie van strobilurinen op secondaire aangrijpingsplaatsen van de middelen. Zo zou overexpressie van MgMfs1 ervoor kunnen zorgen dat membranen normaal blijven functioneren in de aanwezigheid van strobilurinen. Dit zou van belang kunnen zijn voor het behoud van fitness van strobilurine-resistente stammen.

In **hoofdstuk 5** wordt de klonering en functionele karakterisering beschreven van het ABC transporter gen *MgAtr7*. MgAtr7 vertoont grote homologie met schimmel ABC transporters die een rol spelen bij de gevoeligheid voor azool fungiciden. *MgAtr7* codeert voor een nieuw type hybride ABC transporter waarvan de N-terminus een motief bevat dat kenmerkend is voor een dityrosine/pyoverdine biosynthese eiwit. Op

grond hiervan wordt MgAtr7 beschouwd als het eerste lid van een nieuwe klasse van schimmel ABC transporters die behalve een transporter deel ook een biosynthese deel bezit. Dit type transporter blijkt vooralsnog alleen aanwezig te zijn in *M. graminicola* en *Fusarium graminearum*. Functionele analyses toonden aan dat dit gen noch bescherming biedt tegen fungiciden noch betrokken is bij virulentie op tarwe, maar wel een rol speelt bij het handhaven van ijzerhomeostase.

Hoofdstuk 6 beschrijft de resultaten van experimenten met middelen, waarvan bekend is dat ze kunnen functioneren als modulatoren van ABC transporters. Getest werd of deze middelen de fungitoxiciteit van het azool fungicide cyproconazool tegen *M. graminicola* kunnen versterken en de ontwikkeling van de septoria bladvlekkenziekte op tarwezaailingen kunnen onderdrukken. De resultaten beschreven in dit hoofdstuk tonen aan dat sommige verbindingen *in vitro* synergistische werking vertonen met de activiteit van cyproconazool en de efflux van het fungicide uit schimmelcellen kunnen tegengaan. *In planta* werd echter geen synergistische activiteit tussen de modulatoren en cyproconazool gevonden. Enkele van de geteste verbindingen zijn *in vitro* vrijwel niet toxisch voor *M. graminicola* maar hebben wel een significante ziektewerende werking op tarwezaailingen in zowel preventieve als curatieve spuitproeven. De resultaten suggereren dat dergelijke verbindingen een indirecte werking bezitten door modulatie van ABC transporters die betrokken zijn bij virulentie van schimmels. De ontdekking van zulke modulatoren kan leiden tot de ontwikkeling van een nieuwe klasse van middelen met een ziektewerende werking.

In **Hoofdstuk 7** worden de resultaten verkregen in dit proefschrift bediscussieerd met speciale nadruk op geselecteerde onderwerpen, zoals de buitengewoon brede substraat specificiteit van de multidrug transporter MgMfs1, de rol van de transporter in strobilurine gevoeligheid en resistentie in veldisolaten van *M. graminicola*, de structuur van de ABC transporter MgAtr7 en zijn functie in ijzer-siderofoor transport en de potentie van ABC transporter modulatoren als leidraad bij de ontwikkeling van nieuwe ziektewerende middelen.

Samenvattend tonen de gegevens die in dit proefschrift beschreven worden aan dat de MFS transporter MgMfs1 van *M. graminicola* een belangrijke rol speelt in gevoeligheid en resistentie voor fungiciden, in het bijzonder voor strobilurinen. Verder wordt een nieuw type ABC transporter met een nog niet eerder beschreven functie in

ijzer homeostase onderzocht. Tenslotte hebben we aangetoond dat geneesmiddelen met een ABC transporter modulerende werking een indirecte werking tegen plantenziekten kunnen bezitten en dat zulke modulatoren als leidraad kunnen dienen bij de ontwikkeling van nieuwe ziektewerende middelen.

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My dear mother and father. In addition to what very kind parents do for their children, you, as professional teachers, started my official education right after my

birth! Science, the road that I have chosen to reach my ideals, was also your choice for me. I am amazed how much you suffered and what tricks you used to keep me passionate on this rocky road. But, no worries anymore since the taste of science in my mouth is now sweet enough to automatically keep me going this way. My lovely mother, your children's happiness was your most enjoyable joy. You sacrificed your comfort, your youth, and your life, in one word 'yourself', for your children. It is absolutely obvious that I can never compensate even a drop of the ocean of your favors. Based on our culture, assuming that kissing hands is one of the ultimate symbols of appreciation, I am very proud and honored to kiss your tired hands and feet. For me, the words 'pure love', 'real sacrifice' and 'absolute devotion' give the best match with the word 'mother'.

Maarten and Lute, my very honorable 'co-promotors'. To have you both as supervisors in such a luxurious Ph.D. position can not be justified by saying "I was lucky", or even more than lucky! Most likely, I did a very good deed in my life to be rewarded such remunerations. A combination of a very well-experienced and a fresh teacher, globally well known in their own field with valuable publications and both very kind. After experiencing different academic environments as a student and listening to stories from friends, especially Iranian Ph.D. students studying and working in different departments of Wageningen UR, other universities in the Netherlands, in Iran and all other parts of the world, I have enough data to give a good judgment on you. You are just perfect and wonderful! Big scientists with great personalities. My Ph.D. study would not have been successfully completed without your endless kindness and help. During this period, I received your continuous scientific, social and emotional support which was highly appreciated. Maarten, you are very well-organized and punctual, I think even more than the Dutch standards. I never had to wait long for my corrected manuscripts. My experiments and progress were continuously checked by you. You carefully looked at the results, encouraged me with the good ones, guided me to solve problems, and to cope with good time management. You always urged me to come to your office, to phone you at home, or to contact you in case of any question and problem, whenever needed. Oh yeah, a luxurious position! In addition to all your kindness, your mediation for financial support of the last part of my project gave me the required comfort to complete the thesis, which is highly appreciated. Mahnaz and I have

enjoyed your warm hospitality at your very neat and lovely place several times at different occasions, which we appreciate. The high talent of your esteemed spouse Bea is always remembered by us. As far as I can think, in addition to her direct kindness, she has a big role in all the supports that I got from you. Maarten, I am happy that I had opportunity to profit from your supervision just before your retirement and before the closure of the ABC lab. I am really honored to be recorded as your last Ph.D. student in the history of your life. All the best and I wish you a long-lasting, healthy and happy life with Bea in your retirement. Lute, you were supposed to be my daily supervisor. Actually, you were not! In fact, you acted as my supervisor every hour and minute! Since you were always in my neighbourhood, my questions were more easily and quickly answered than via internet. You taught me the fundamentals of molecular genetics and fungal transporters and trained me their practical aspects. For the entire experimental part of my Ph.D. program your eyes were on me and my work, as we worked together in the ABC lab until the very end of its life as the last ABC veterans. My manuscripts were also quickly modified in your hands. Translation of my Dutch letters and documents frequently disturbed you but I am happy that you, as a close friend, were aware of what was requested from me and what was going to happen to me already before I knew! In this way, the sharpness of shocking news could be moderated by you. Anyway, in the history of your professional life, I am recorded as your first Ph.D. student. I hope that the next students will be much easier to deal with than me! I wish you all the best with your new career at CBS. Your respected spouse Karin is also appreciated for her kindness to me and Mahnaz. Maarten and Lute, you are now much more for me than ‘co-promotors’, supervisors, teachers, scientists, and colleagues. You are my dear friends and relatives, symbols of ethics, and examples of perfect human beings whose names will immediately remind me to The Netherlands. How lucky a country can be to have such representatives in hearts and minds?

Pierre, my respected ‘promotor’ and head of Department of Phytopathology. Your active role and wise strategies to lead and direct the lab is the bases for the high standing publication record, success, and honor of the Laboratory. I am pleased that I could spend several precious years of my life in such a group. Despite the very busy schedule, you were easily accessible to all Ph.D. students to discuss ideas and solve problems. Love for science can be really found in you. Your quick proof-reading and

critical comments to increase the quality of my papers and chapters of my thesis are appreciated. Sending documents by Ph.D. students at 21:58 h for correction may look O.K. but seeing them back only a couple of hours later at 00:26 h, although highly appreciated, worries me. You should remain healthy not only for yourself and your esteemed family, but also for the international Phytopathological community. I would advice you “take care of your health”!

Working in the ABC lab was a pleasure to me. There, I got to know some wonderful people. Ioannis, when I arrived you were busy with the last steps of your Ph.D. study. This was a good opportunity to get extra theoretical and practical help in the beginning of my project. Subsequently, you became a postdoc while I was doing the last parts of my work. Again, you functioned as a source of knowledge and experience to help me in finalizing my thesis. You gave priority to my questions rather than to your own work. Our joined trip to Kiel was memorable and very enjoyable. Thank you so much for everything. Your Greek nationality encouraged me to dive into the history and highlighted me the similarities between the ancient civilizations of Greece and Iran. Those two bright parts of human history are really precious treasures proving the magnificence and glory of humanity. Hans, you instructed me in the lab and helped me with some of my accumulation experiments. Those experiments have increased the quality of my thesis. Thank you. Aurelie, you are my former roommate and M.Sc. student of the ABC group. Maarten’s idea, your excellent work and my experiments showed the potential of modulators as disease control agents. It is absolutely worthwhile to continue this study, if possible. Thank you and good luck with the rest of your Ph.D. study at the Max Planck Institute, Germany. Other former M.Sc. students of the ABC group are Melanie, Paul and Rose. I really enjoyed working together with you. Some parts of your work have also been incorporated in this thesis. I appreciate your friendship, support and contribution, and hope you are successful in your career. Ciska, a former technician of the ABC group, shared with me the office for a period of time. You answered quickly my questions related to the Dutch culture. Thanks for your kindness, help and hospitality. Olivier, your presence at the ABC lab as an Erasmus M.Sc. student was short, but I enjoyed our late evening social and cultural talks. Alan, Henk-jan and Keisuke are great ABC researchers. Although my working period did not

coincide with your presence in the group, I really appreciate the opportunities to meet you and to learn your great personalities.

I appreciate the monthly discussions within the Dutch Mycosphaerella Group including researchers of PRI and Phytopathology from Wageningen UR and CBS from Utrecht. Gert, you have been very kind and supportive throughout my study. You are globally acknowledged for your expertise in *Mycosphaerella* research. It is an honor to have a joined paper with you. For three months, I worked at PRI to obtain *Mycosphaerella graminicola* isolates. Furthermore, I and my wife enjoyed your hospitality. Thank you and your respected spouse. Pedro, your passion in fungal and *Mycosphaerella* research is really special. Within the group, I appreciate research discussions with Cees, Els, Ineke, Odette, Rahim, Sarah, Sarrah, and Theo from PRI and Edwin, Ewald, Marizeth, and Mahdi from CBS. I thank Rients (Department of Plant Breeding, WUR) for his impact on my work, and his supportive and warm personality.

I thank the staff members of the Department of Phytopathology Bart, Francine, Jan, Jos, and Matthieu for their scientific advice and support. I would like to appreciate my multinational Phytopathology friends and colleagues Ahmed, Andre, Andrea, Corrie, Emilie, Ester, Grardy, Ha, Harold, Ilona, Irene, Iris, Jan-Kees, John, Jorge, Jun, Klaas, Lia, Marco, Maria, Martijn, Mieke, Orlando, Peter (3x), Pieter, Qing, Rays, Rob, Rodrigo, Sander, Suzan, Ursula, Wladimir, and Yaita for their great support and the wonderful and friendly environment they created. I also thank Ali and Ria, the helpful secretaries of Phytopathology. I am very pleased that I could spend a part of my life in the tiny town Wageningen and in The Netherlands, a small country with great people. The Dutch, people with smiley faces and kind hearts.

Years of life as a Ph.D. student far from my home country and relatives were as enjoyable as being at home. I did not really feel home-sick since I enjoyed a warm and friendly community of Iranian students in Wageningen. The ceremonies we had, were always memorable. Thank you and the esteemed families for kind help and assistance. It was a great chance to have my old friends Hossein and Rahim around me in Wageningen. Our detailed talks about the same scientific topics, former teachers, colleagues, old memories, affiliations and future engagement were opportunities to feel home. I appreciate your support and advice. Mostafa, Hossein and Rahim, our weekly Saturday night family parties provided valuable moments to discuss our current

scientific and social issues and our country. I hope to keep such strong ties, friendship and collaborations in future.

The financial support of my Ph.D. study in the Netherlands was provided by the Agricultural Research and Education Organization (AREO, Ministry of Agriculture) of Iran and the Iranian Ministry of Science, Research, and Technology. I also like to thank my dear friends and colleagues in Iran Eng. Mehran Patpour (Cereal Pathology Research Unit, SPII), Eng. Hassan Ebrahimi, and Eng. Mashallah Belbasi (Faculty of Agriculture, University of Tehran) for their efforts to make my study in The Netherlands possible.

My kind sister Halleh. Despite your own busy schedule you handled a lot of my administrative affairs during my stay in the Netherlands. I appreciate that and hope that your lovely family will have a very healthy and happy life. My modest brother, Farzin. I always appreciate your expertise in agricultural irrigation engineering and your broad knowledge in general. During my family speeches, you smartly note, remind and correct my errors, and then make fun out of them! I am delighted to remember these stories, and to rely on your general knowledge if needed. My little sister and brother, please forgive my faults for the fact that 'I am a big brother'! The family of my parents-in-law, The Seyyed Hejazie. Your encouragement is acknowledged as a permanent driving force that will guarantee my presence in science for ever. Finally, I would like to express my deep gratitude to all people, and cultural and social values involved in my education from the very beginning. These elements had a precious impact on my personal life by directing me in the arena of science where I found my joy and satisfaction. I am fully aware that I owe these achievements to my fabulous home-province Azarbayjan and my lovely country Iran.

Ramin Rookparvar

Wageningen, The Netherland
February 2007

رامین روح پرور

واخنینگن، هلند
اسفند ۱۳۸۵

About the author

Ramin Roohparvar was born in 1969 in Tabriz, one of the five most important cities of Iran, and the capital of East Azarbayjan province, a mountainous area with fabulous four seasons located in the northwest of Iran. He followed primary and secondary schools and subsequently high school in biological sciences in Tabriz. Passing the Iranian national entrance exam for governmental universities in 1988, he was accepted in the Plant Protection Group, Faculty of Agriculture, University of Tabriz, where in September 1992 he received his B.Sc. degree with distinction in the field of Agricultural Engineering-Plant Protection. As a part of his obligatory military service, he was offered the opportunity to work in the Agricultural Research Center of East Azarbayjan on weed control for 19 months. After completion of military service in 1995 and wishing to experience different scientific environments for the continuation of his study and research, he successfully passed the national M.Sc. exam, and entered the Department of Plant Pathology, Plant Protection Group, Faculty of Agriculture, University of Tehran (the mother university of Iran), Karaj. During this period, he was granted a scholarship of the Agricultural Research and Education Organization (AREO) of Iran, Ministry of Agriculture, and was affiliated at one of its mother institutes, the Seed and Plant Improvement Research Institute (SPII). His M.Sc. research project resulted in a M.Sc. degree with distinction in the field of Agricultural Engineering-Plant Pathology in September 1998. Results of this thesis was presented at several national and international conferences and published as a paper entitled 'Induced chitinase activity in resistant wheat leaves inoculated with an incompatible race of *Puccinia striiformis* f. sp. *tritici*, the causal agent of yellow rust disease' in Mycopathologia. He was then appointed as an academic staff member of AREO and started his work as a pathobreeder on wheat leaf rust in the Cereal Pathology Unit, Department of Cereal Research, SPII headquarter, Karaj, Iran. Passing an additional national exam, he was awarded a scholarship to do a Ph.D. abroad with financial support of AREO and the Ministry of Science, Research and Technology (MSRT) of Iran. As he wished to expand his knowledge in molecular aspects of cereal-fungal pathogen relationships, disease control and fungicide resistance, in June 2002, he joined the Laboratory of

Phytopathology, Wageningen University and Research Center, Wageningen, the Netherlands, where he conducted his Ph.D. study on drug transporters of the fungal wheat pathogen *Mycosphaerella graminicola*. Results of his Ph.D. research have been presented at several international conferences and have been published in or submitted to peer-reviewed journals as listed on the next page. Furthermore, he received the education certificate from the Graduate School 'Experimental Plant Sciences' (EPS).

In February 26, 2007, he received his Ph.D. degree and during his Ph.D. he gained valuable knowledge and experience in molecular plant pathology. As of this date, he will continue his scientific career as an assistant research professor in the Department of Cereal Research at SPII, Karaj, Iran.

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List of publications

Peer-reviewed articles:

Mohammadi, M., **Roohparvar, R.,** Torabi, M., 2002. Induced chitinase activity in resistant wheat leaves inoculated with an incompatible race of *Puccinia striiformis* f.sp. *tritici*, the causal agent of yellow rust disease. Mycopathologia 154, 119-126.

Roohparvar, R., De Waard, M.A., Kema, G.H.J., Zwiers, L.-H., 2006. MgMfs1, a major facilitator superfamily transporter from the fungal wheat pathogen *Mycosphaerella graminicola*, is a strong protectant against natural toxic compounds and fungicides. Fungal Genetics and Biology. In press, doi:10.1016/j.fgb.2006.09.007.

Roohparvar, R., De Waard, M.A., Zwiers, L.H., 2007. Major facilitator superfamily (MFS) transporters and their significance in plant pathogenic fungi. Submitted.

Roohparvar, R., Huser, A., Zwiers, L.-H., Waard, M.A., 2007. Control of *Mycosphaerella graminicola* on wheat seedlings by medical drugs known to modulate the activity of ATP-binding cassette transporters. Submitted.

Roohparvar, R., Mehrabi, R., Van Nistelrooy, J.G.M., Zwiers, L.-H., De Waard, M.A., 2007. The drug transporter MgMfs1 affects sensitivity of the fungal wheat pathogen *Mycosphaerella graminicola* to the strobilurin fungicide trifloxystrobin. Submitted.

Zwiers, L.-H., **Roohparvar, R.,** De Waard, M.A., 2006. MgAtr7, a new type of ABC transporter from *Mycosphaerella graminicola* involved in iron homeostasis. Fungal Genetics and Biology. Accepted.

Proceedings and abstracts:

Roohparvar, R., Zwiers, L.-H., Kema, G.H.J., De Waard, M.A., 2003. *MgMfs1*, a major facilitator superfamily transporter gene of *Mycosphaerella graminicola* encodes a potent multidrug transporter. In: pp. 77-82, Global Insights into the Septoria and Stagonospora Diseases of Cereals. G.H.J. Kema, M. Van Ginkel, M. Harrabi (Eds.), Proceedings of the 6th International Symposium on Septoria and Stagonospora Diseases of Cereals. Tunis, Tunisia.

Roohparvar, R., Zwiers, L.-H., Kema, G.H.J., De Waard, M.A., 2005. Multidrug resistance in *Mycosphaerella graminicola* involves the novel major facilitator superfamily (MFS) transporter gene *MgMfs1*. In: p. 136, Proceedings of the 23rd Fungal Genetics Conference. Asilomar, California, USA.

Zwiers, L.-H., **Roohparvar, R.,** De Waard, M.A., 2006. The *Mycosphaerella graminicola* ABC transporter *MgAtr8* is a dicistronic messenger involved in iron homeostasis. In: p. 329, Proceedings of the 8th European Conference on Fungal Genetics. Vienna, Austria.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Ramin Roohparvar
Date: 26 February 2007
Group: Phytopathology, Wageningen University

1) Start-up phase	date
► **First presentation of your project**	
Functional analysis of virulence genes from *Mycosphaerella graminicola*	Nov 1, 2002
► **Writing or rewriting a project proposal**	
Functional analysis of virulence genes from *Mycosphaerella graminicola*	Jun-Sep 2002
► **Writing a review or book chapter**	
Major facilitator superfamily (MFS) transporters and their significance in plant pathogenic fungi	Aug-Nov 2006
► **MSc courses**	
Molecular aspects of bio-interactions PHP-30806	Sep 1-5, 2003
► **Laboratory use of isotopes**	
Safe handling with radioactive materials and sources	Jan 7-9, 2003
Subtotal Start-up Phase	*15.7 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
8th EPS PhD student day, Utrecht University	Mar 27, 2003
9th EPS PhD student day, Vrije Universiteit Amsterdam	Jun 3, 2004
10th EPS PhD student day, Radboud University, Nijmegen	Jun 2, 2005
11th EPS PhD student day, Wageningen University	Sep 19, 2006
► **EPS theme symposia**	
Theme II 'Interactions between Plants and Biotic Agents', Vrije Universiteit Amsterdam	Jan 10, 2003
Theme II 'Interactions between Plants and Biotic Agents', Utrecht University	Sep 17, 2004
► **NWO Lunteren days and other National Platforms**	
Willie Commelin Scholten Day, Phytopathology section, Utrecht University	Jan 30, 2003
ALW platform Molecular Genetics, Lunteren	Nov 3-4, 2003
Willie Commelin Scholten Day, Phytopathology section, Utrecht University	Jan 22, 2004
NWO-ALW annual meeting, Lunteren	Apr 5-6, 2004
NWO-ALW annual meeting, Lunteren	Apr 4-5, 2005
ALW platform Molecular Genetics, Lunteren	Oct 13-14, 2005
► **Seminars (series), workshops and symposia**	
The KNBV ceremony and symposium of the Hugo de Vries-prijs, Lunteren	Sep 27, 2002
Seminar 'Cytogenetics in filamentous fungi', Dr. M. Taga, Wageningen	Nov 28, 2002
Seminar 'Investigating the biology of plant infection by the rice blast fungus', Prof. N. J. Talbot, Wageningen	Apr 29, 2003
Flying seminar 'The molecular control of specific pathogen recognition by plants', Prof. J. Dangle, Wageningen	May 9, 2003
Seminar 'Micro-array application', Dr. R. Evers, Wageningen	Jun 24, 2003
CBS/Wageningen Phytopathology symposium, Utrecht	Jun 27, 2003
CBS/Wageningen Phytopathology symposium 'Phylogenomics of Fungi', Utrecht	May 10, 2004
Genomics momentum 2004: Genomics for our world, Rotterdam	Aug 30-Sep 1, 2004
Symposium on 'Systems biology' in honor of Prof. Pierre de Wit, Wageningen	Nov 4, 2004
Phytopathology research day, Wageningen	May 18, 2005
Seminar 'Management of phytophthora blight of cucurbits and peppers', Dr. M. Babadoost, Wageningen	Jul 18, 2005
Seminar 'Reprogramming the host: The effector secretome of *Phytophthora infestans* ', Prof. S. Kamoun, Wageningen	Oct 5, 2005
► **Seminar plus**	
► **International symposia and congresses**	
6th International Symposium on Septoria/Stagonospora Diseases of Cereals, Tunis, Tunisia	Dec 8-12, 2003
56th International Symposium on Crop Protection, Ghent, Belgium	May 4, 2004
23rd Fungal Genetics Conference, Asilomar, CA, USA	Mar 15-20, 2005
► **Presentations**	
Poster presentation, ALW platform Molecular Genetics, Lunteren	Nov 3-4, 2003
Oral presentation, 6th International Symposium on Septoria/Stagonospora Diseases of Cereals, Tunis, Tunisia	Dec 8-12, 2003
Oral presentation, Willie Commelin Scholten day, Phytopathology section, Utrecht University	Jan 22, 2004
Poster presentation, 23rd Fungal Genetics Conference, Asilomar, CA, USA	Mar 13-22, 2005
Poster presentation, 10th EPS PhD student day, Radboud University of Nijmegen	Jun 2, 2005
Oral flash presentation, ALW platform Molecular genetics, Lunteren	Oct 13-14, 2005
Poster presentation, 8th European Conference on Fungal Genetics, Viena, Austria	Apr 8-11, 2006
► **IAB interview**	Jun 2, 2005
► **Excursions**	
Subtotal Scientific Exposure	*17.0 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Autumn School 'Disease Resistance in Plants', Wageningen University	Oct 14-16, 2002
Summer School 'Functional Genomics: theory and hands-on data analysis, Utrecht University	Aug 25-28, 2003
► **Journal club**	
Weekly ABC group discussion	2002-2006
Weekly Phytopathology lab meeting	2002-2006
Monthly Dutch Mycosphaerella Group discussion	2002-2006
► **Individual research training**	
Subtotal In-Depth Studies	*5.1 credits**

4) Personal development	date
► **Skill training courses**	
Self-study English course, University language center (CENTA), Wageningen	Autumn 2002
Guide to digital scientific artwork, Wageningen University	Dec 14-15, 2004
Seminar 'Ins and outs of printing a PhD thesis', Wageningen	Oct 6, 2005
Working with EndNote 8, Wageningen University	Nov 17, 2005
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**40.8**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

The research described in this thesis was performed at the Laboratory of Phytopathology of Wageningen University and Research Center, Wageningen, The Netherlands, under the Training and Supervision Plan of the Graduate School ‘Experimental Plant Sciences’.

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Cover front page: Disease development of septoria leaf blotch on wheat. Background picture: Experimental wheat field equipped with mist sprayers at the Cereal Research Department, SPII, Karaj, Iran. Picture: Mehran Patpour

Cover back page: The river Rhine bathed under the morning sun. View from the botanical garden of Wageningen University, Wageningen, The Netherlands. Picture: Maarten de Waard

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شده توسط این ژن (MgAtr7) نوع هیبریدی جدیدی از ترابر ABC با وضعیت [1] $[NBF\text{-}TM]_2$ می باشد که در انتهای آمینی دارای یک موتیف [2] ویژه ای است که در پروتئین بیوسنتز دیتیروزین\پیووردین دیده می شود. این پروتئین اولین عضو گروه جدیدی از ترابرهای ABC محسوب می شود که شامل هر دو بخش ترابری و بیوسنتزی می باشند. بررسی های عملکردی نشان دادند که این ژن در حساسیت به قارچکشها و بیماریزایی بر روی گندم دخالت نداشته، لیکن در حفظ تعادل آهن نقش دارد.

فصل ششم توانایی داروهای پزشکی که به عنوان تنظیمگرهای ترابرهای ABC شناخته شده اند را در تشدید فعالیت قارچکش آزول سیپروکونازول بر علیه رشد درون شیشه ای *M. graminicola* و نیز در کنترل پیشرفت بیماری حاصله بر روی گیاهچه های گندم تشریح می نماید. در این فصل نشان داده شده است که برخی از این ترکیبات می توانند در شرایط درون شیشه ای، فعالیت سیپروکونازول را تشدید نموده و از فعالیت سلولهای قارچی در دفع این قارچکش جلوگیری نمایند. با این وجود، برهمکنشهای تشدید کنندگی [3] بین تنظیمگرها و سیپروکونازول در شرایط درون گیاهی مشاهده نگردید. برخی از این ترکیبات که در حالت درون شیشه ای به صورت بالقوه فاقد هر گونه فعالیت سمی نسبت به *M. graminicola* بودند، در آزمایشات سمپاشی پیشگیری کننده و درمان کننده فعالیت معنی داری در کنترل بیماری بر روی گیاهچه های گندم نشان دادند. نتایج به دست آمده نشان می دهد که این ترکیبات می توانند از طریق تنظیم ترابرهای ABC موثر در بیماریزایی دارای یک فعالیت غیرمستقیم کنترل بیماری باشند. چنین تنظیمگرهایی ممکن است گروه جدیدی از ترکیبات کنترل کننده بیماری را تاسیس نمایند.

فصل هفتم ضمن تاکید ویژه بر روی برخی عناوین، بحث کلی رساله حاضر را ارائه می نماید. این عناوین عبارتند از: دامنه بسیار وسیع سابستریت برای ترابر چندارویی MgMfs1، نقش این ترابر در حساسیت و مقاومت *M. graminicola* نسبت به استروبیلورینها، عملکرد MgAtr7 به عنوان ترابر ABC در انتقال آهن-سیدروفور و پتانسیل تنظیمگرهای ترابرهای ABC به عنوان ترکیبات مقدماتی در مسیر کشف مواد کنترل کننده بیماری.

داده های ارائه شده در این رساله نشان می دهد که MgMfs1 به عنوان اولین ترابر دارویی MFS کلون شده *M. graminicola*، نقش مهمی در حساسیت و مقاومت به قارچکشها بویژه قارچکشهای استروبیلورین دارد. همچنین، نوع جدیدی از ترابر دارویی ABC با عملکرد جدیدی در حفظ تعادل آهن کشف گردید. بعلاوه، ما نشان دادیم که داروهای پزشکی تنظیمگر ترابرهای ABC، می توانند دارای فعالیت غیرمستقیم کنترل بیماری بوده و ممکن است گروه جدیدی از ترکیبات کنترل کننده بیماریهای گیاهی را تاسیس نمایند.

[1] Configuration
[2] Motif
[3] Synergistic interactions

همچنین نقش ترابرهای MFS شناخته شده در قارچهای بیماریزای گیاهی در دفع و ترشح انواع مختلف ترکیبات ضدقارچی، MDR و بیماریزایی مرور می گردد. در انتها تاثیر ترابرهای دارویی MFS قارچها بر روی حساسیت پایه به قارچکشها، MDR و کشف ترکیبات جدید مورد بحث قرار می گیرد.

فصل های سوم و **چهارم** بر روی شناسائی و بررسی عملکرد [1] ژن *MgMfs1* به عنوان اولین ژن ترابر MFS کلون شده از *M. graminicola* با شباهت [2] بالا نسبت به ترابرهای MFS قارچی موجود در خانواده DHA2، و دخیل در ترشح سموم قارچی و MDR تمرکز دارند. نتایج موجود در **فصل سوم** بر عدم دخالت MgMfs1 در بیماریزایی بر روی گندم دلالت دارد. با این وجود انتقال و بیان [3] این ژن در قارچ مخمر و بررسیهای توصیفی-فنوتیپی [4] موتانتهای *M. graminicola* حامل ژن غیر فعال شده *MgMfs1*، نشان می دهد که این ترابر در شرایط درون شیشه ای [5] می تواند به عنوان یک ترابر بسیار قوی چندگانه دارویی با توان انتقال دامنه وسیعی از مواد شیمیایی مانند سموم قارچی، متابولیتهای گیاهی و قارچکشها بویژه استروبیلورینها [6] عمل نماید. در **فصل چهارم** نشان داده شده است که MgMfs1 در شرایط درون شیشه ای و درون گیاهی [7] ، نقش مهمی در حساسیت *M. graminicola* به قارچکش استروبیلورین تریفلوکسیستروبین [8] ایفا می نماید. دفع قارچکشهای استروبیلورین در موتانتهای حامل ژن غیر فعال *MgMfs1*، کاهش یافته و آزمایشات کنترل بیماری روی گیاهچه های گندم حاکی از افزایش حساسیت این موتانتها نسبت به قارچکش تریفلوکسیستروبین می باشد. **فصل چهارم** همچنین نشان می دهد که علاوه بر نقش عمده موتاسیون نقطه هدف [9] G143A در ژن سیتوکروم بی [10] در مقاومت استرینهای مزرعه ای به استروبیلورینها، بیش بیان [11] *MgMfs1* ممکن است به عنوان یک مکانیسم حفاظتی اضافی عمل نماید. بیش بیان این ژن احتمالا از ملزومات نگهداری مناسب عملکرد غشا [12] و بقا و سازگاری [13] طبیعی این استرینها در حضور استروبیلورینها می باشد.

فصل پنجم به شرح کلون سازی مولکولی و توصیف عملکرد *MgAtr7* به عنوان یک ژن ترابر ABC با شباهت بالا نسبت به ترابرهای ABC قارچی دخیل در حساسیت به قارچکشهای آزول [14] و ظاهرا منحصر به *M. graminicola* و *Fusarium graminearum* می پردازد. پروتئین کد

[1] Functional analysis
[2] Homology
[3] Heterologous expression
[4] Phenotypic characterization
[5] *In vitro*
[6] Strobilurins
[7] *In planta*
[8] Trifloxystrobin
[9] Target site mutation
[10] Cytochrome b
[11] Overexpression
[12] Proper maintenance of membrane functioning
[13] Fitness
[14] Azole

خلاصه

گندم با اختصاص ۱۷٪ از سطح زیر کشت جهانی و به عنوان عمده ترین منبع غذایی برای ۳۵٪ جمعیت جهان، مهمترین محصول غله به شمار می رود. در ایران، گندم مهمترین محصول کشاورزی بوده و نان به عنوان عمده ترین مورد مصرف آن بیش از ۵۰٪ کالری روزانه را تامین می نماید. قارچ آسکومیست *Mycosphaerella graminicola* (Fuckel) J. Schroeter عامل بیماری سپتوریوز برگی[1] گندم، بویژه در مناطقی که در طول فصل رشد دارای میزان بارش بالا هستند، بتدریج به عنوان یکی از خسارتزاترین پاتوژنهای اندامهای هوایی گندم ظاهر گردیده است. مدیریت این بیماری عمدتا با استفاده از ارقام مقاوم، عملیات زراعی و کنترل شیمیایی انجام می گیرد. استفاده گسترده از انواع مختلف قارچکشها در کنترل بیماری، سبب تحمیل فشار انتخاب[2] بر روی جمعیت این پاتوژن و در نتیجه منجر به ظهور استرینهای[3] مقاوم به قارچکش گردیده است.

رساله حاضر به بررسی نوع خاصی از پروتئینهای غشای سلولی *M. graminicola* که در دفع و ترشح مواد شیمیایی دخالت دارند، می پردازد. این پروتئینها که به ترابرهای دفعی دارویی[4] معروفند، وابسته به دو گروه ترابرهای 'بزرگ خانواده' تسهیلگرهای اصلی[5] (MFS) و کاستهای قابل اتصال به ATP[6] (ABC) می باشند. در این پژوهش، نقش ترابرهای دفعی دارویی در حساسیت به قارچکشها، ظهور مقاومت چندگانه دارویی[7] (MDR) و بیماریزایی بر روی گندم مورد بررسی قرار گرفته است. همچنین نوع جدیدی از ترابر ABC که در تنظیم تعادل آهن[8] نقش دارد، توصیف گردیده است. مواد بازدارنده[9] ترابرهای دارویی که به عنوان فاکتورهای بیماریزایی شناخته شده اند، ممکن است به عنوان عوامل غیرمستقیم کنترل بیماری عمل نمایند. از این رو، توانایی مواد تنظیمگر[10] ترابرهای دارویی ABC با مصرف پزشکی در کنترل بیماری سپتوریوز برگی بر روی گیاهچه های گندم مورد بررسی قرار گرفت.

فصل اول به ارائه یک نگاه کلی در مورد اهمیت جهانی گندم، سپتوریوز برگی، *M. graminicola* و مدیریت بیماری با تاکید بر کنترل شیمیایی، مقاومت به قارچکشها و نقش ترابرهای دارویی در حساسیت و مقاومت به قارچکشها می پردازد.

فصل دوم به مرور ترابرهای MFS بویژه ترابرهای دارویی و نقش آنها در ارتباط با قارچهای بیماریزای گیاهی اختصاص دارد. این فصل به ارائه یک نگاه کلی در مورد ویژگیهای عمومی ترابرهای MFS شامل طبقه بندی، خصوصیات فیزیکی و مکانیسمهای انتقال می پردازد.

[1] Septoria tritici leaf blotch
[2] Selection pressure
[3] Strains
[4] Drug efflux transporters
[5] Major facilitator superfamily
[6] ATP-binding cassette
[7] Multidrug resistance
[8] Iron homeostasis
[9] Inhibitors
[10] Modulators

ترابرهای دارویی در غشای سلولی پاتوژن قارچی گندم

Mycosphaerella graminicola

رامین روح پرور

رساله دکترای تخصصی

دانشگاه واخنینگن

هلند

اسفند ۱۳۸۵